DOCTORAL THESIS

Development of Improved Methods for Low Template DNA Analysis

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DEVELOPMENT OF IMPROVED METHODS FOR LOW TEMPLATE DNA ANALYSIS

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ABSTRACT

Traditional forensic DNA profiling by PCR of short tandem repeats is considered a robust and reliable method of human identification. However, difficulties can occur when the starting DNA template is limited in quantity or quality. Various methods to improve the sensitivity of the PCR have been suggested for use with samples containing low levels of starting template. While these methods do allow for increasingly smaller amounts of DNA to be examined, stochastic sampling effects seen in the final profiles often make interpretation of results difficult, indicating a need for improved low template DNA analysis and interpretation strategies. This project, therefore, aimed to investigate and develop alternative methods for analysis of samples with limited starting template.

Initial work on this project aimed to assess the current methods of low template DNA analysis. The LCN method used by forensic laboratories involves dividing a low template DNA extract into several replicates, usually three, and generating a consensus profile which includes only alleles seen in two or more of the replicates. It seems counter-intuitive to split an already low amount of DNA into even smaller amounts and so, in this study, the quality of consensus profiles derived through replicate analysis of a low template amount of DNA split into three was compared to profiles obtained using that whole low template DNA extract for a single amplification. Overall, results showed that the consensus profile was less informative than the profile obtained using the entire low template sample, with increased allele and locus drop out observed with replicate analysis. However, by constructing a consensus profile any spurious alleles were eliminated from the final profile. Such additional alleles, in the form of increased stutter or random allele drop in, were sometimes observed in profiles from a single amplification.

This project also examined methods to increase, in a non-exponential manner, the DNA template available for STR PCR analysis. A novel Pre-PCR technique was investigated, where the STR region of interest was first amplified in a linear fashion so that a single new copy of the target was produced with each Pre-PCR cycle. The

resulting product was then subjected to standard PCR analysis. Results showed that more alleles were recovered in each profile with Pre-PCR amplification compared to samples amplified without the Pre-PCR procedure. Furthermore the peak height ratios did not differ greatly in samples with and without Pre-PCR treatment. This indicated that the linear amplification of the Pre-PCR was increasing the number of template copies available for the PCR without introducing substantial amplification bias. This contrasts with additional exponential amplification where there is significant increase in amplification bias observed.

Various whole genome amplification chemistries were also examined as possible methods for increasing the DNA template prior to STR analysis. Modifications to the recommended protocols were also investigated. Results showed that a novel AT WGA kit amplified the DNA in the most representative fashion, with more alleles recovered and higher peak height ratios compared to other commercial WGA kits. Slight improvements were seen in the results when the AT WGA reaction was divided prior to amplification then pooled for STR analysis.

Laser microdissection was used to collect single and small numbers of cells for STR PCR analysis. DNA from the cell samples was amplified using both standard and increased cycle PCR protocols to determine if improved results could be obtained with additional PCR cycles. Cells were also subjected to WGA prior to STR analysis to determine if further improvements could be observed. Various extraction methodologies were examined to determine which best disrupted the cells for WGA. Results showed that best results were achieved using an increased cycle PCR amplification rather than WGA, with a complete STR profile obtained from as few as five buccal cells using a 34-cycle PCR.

In the final study, mitochondrial DNA was analysed as an alternative to autosomal STR markers for forensic identification using low template DNA. Results showed that complete HV1 and HV2 sequences could be obtained using as little as 0.01pg to 0.1pg of genomic DNA. Various WGA techniques were examined, with sequencing

successfully performed on product obtained from all WGA kits when diluted genomic DNA was amplified. Laser microdissected cells were also used as starting template for HV1 sequencing with limited success. A complete HV1 profile was obtained from a single buccal cell when the sample was used directly for mtDNA sequencing. However, amplifying the cells with WGA prior to sequencing was largely unsuccessful.

DECLARATION AND ADDENDUM

This thesis is submitted to Bond University in fulfilment of the requirements of the degree of Doctor of Philosophy by Research.

This thesis represents my own original work towards this research degree and contains no material which has been previously submitted for a degree or diploma at this University or any other institution, except where due acknowledgement is made.

Kelly Grisedale

February 2014

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ABBREVIATIONS

A	Adenine
ADO	Allele Drop Out
CCD	Charge Coupled Device
C	Cytosine
CE	Capillary Electrophoresis
DNA	Deoxyribonucleic Acid
ESR	Environmental Science and Research Laboratory
FSS	Forensic Science Service
G	Guanine
LCN	Low Copy Number
LDO	Locus Drop Out
LMD	Laser Microdissection
LOD	Limit of Detection
LTDNA	Low Template DNA
MDA	Multiple Displacement Amplification
mtDNA	Mitochondrial DNA
mtWGA	Mitochondrial Whole Genome Amplification
NFI	Netherlands Forensic Institute
OCME	Office of the Chief Medical Examiner
PCR	Polymerase Chain Reaction
PHR	Peak Height Ratio
SGM	Second Generation Multiplex
RFU	Relative Fluorescence Units
STR	Short Tandem Repeat
SWGDAM	Scientific Working Group on DNA Analysis and Methods
T	Thymine
WGA	Whole Genome Amplification

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Since its first use in a forensic setting in 1986 [1], DNA profiling has become a powerful investigative tool and a compelling form of evidence when presented in court. DNA profiling can be used to identify victims of crime, and can also link a suspect to a crime scene or a suspect to a victim due to its ability to provide essentially unique personal identification. One of the main limitations of existing forensic DNA profiling techniques lies in the minimum quantity and quality of DNA fragments that can be examined. Forensic samples may contain only small amounts of DNA or be degraded into small fragments. This can result in the failure of DNA profiling techniques to produce a profile all together, or a reduction in quality of the profile obtained. Issues associated with DNA profiling from low amounts of starting template, known as stochastic effects, make interpretation of profiles difficult [2-6]. Many forensic samples contain DNA from more than one person and these mixed samples are extremely challenging to resolve when working with low levels of DNA due to the inherent stochastic effects.

The current methods of DNA profiling which utilise the Polymerase Chain Reaction (PCR) and capillary electrophoresis (CE) are extremely sensitive, allowing for highly discriminative DNA profiles to be obtained from as little as thirty human cells, or approximately two hundred picograms of DNA [7-13]. However due to the sensitivity of the technique there has come an increased desire to profile even smaller amounts of DNA, in what is termed Low Copy Number (LCN), or Low Template DNA (LTDNA) analysis [14]. This project aims to investigate the reliability of current methods of LTDNA typing and generate novel methods which can overcome the inherent issues associated with working with low amounts of DNA template. Much of the current research in LTDNA analysis is aimed at improving STR profile interpretation strategies. This project aims to reduce stochastic effects such that improved interpretation strategies are not necessary, and LTDNA profiles can be analysed in the same manner as conventional DNA profiles.

This chapter will briefly describe the biology of DNA and review the current practices of forensic DNA profiling. This chapter will then give an in-depth discussion on Low Template DNA profiling, including the various methods used to perform the analysis, validation and interpretation issues, challenges to LTDNA analysis and current and future techniques that can be utilised to avoid the inherent issues of LTDNA typing.

1.2 Biology of DNA

Almost every cell in the human body contains genetic material inherited from both parents. This genetic material, called deoxyribonucleic acid (DNA), is condensed and packaged into structures called chromosomes, which are found in the nucleus of the cell. The fundamental units of heredity, the genes, can be found at specific chromosomal locations, or loci. The human cell contains twenty-three pairs of chromosomes, with one member of each pair inherited from the mother, and the other from the father. The twenty-third pair consists of the sex chromosomes, with females having a pair of X chromosomes and males having one X and one Y chromosome. Therefore, with the exception of X- and Y-linked genes in male cells, each cell contains two copies of every gene, which influence the same trait, but are not necessarily identical. These different versions of the same gene are called alleles.

Within the chromosome, DNA forms a double helix, where two complementary strands join together to form a ladder-like structure. Each strand of the helix is composed of subunits called nucleotides, each of which consists of a deoxyribose sugar molecule, a phosphorous containing group and a nitrogen containing molecule called a base. There are four different bases that can be used to form a nucleotide: adenine (A), thymine (T), cytosine (C), or guanine (G). The two strands in the double helix are joined by these bases in a very specific manner; A can only bind to T, and C can only bind to G, in a relationship known as complementary base pairing. The human genome contains approximately three billion base pairs, and it is the specific sequence of these base pairs that determines the role of the DNA molecule, coding for approximately thirty thousand genes. However, this coding DNA only comprises approximately two per cent of the

total human genome [15, 16]. The remaining ninety-eight per cent consists of non-coding DNA, and it is this non-coding DNA that is the focus of forensic DNA analysis.

Genetic variation between individuals can occur as changes to the sequence at particular base positions or as differences in the sequence length. Individuals are thought to have millions of single base changes, known as single nucleotide polymorphisms (SNPs) [15, 16]. Such changes can include substitutions, insertions or deletions. SNPs have potential as forensic markers since they can give information of an individual's identity, lineage, ancestry and phenotype [17].

Differences in sequence length generally occur in repeated segments of DNA. Approximately 50% of the human genome is made up of repetitive elements [16]. One class of these repetitive elements, known as simple sequence repeats (SSRs), make up approximately 3% of the total DNA and have shown a high degree of length polymorphism in the human population [16]. SSRs can be divided into two broad categories: minisatellites, which have a core repeat unit of 14 to 500 bases; and microsatellites, which have a core repeat unit of 1 to 13 bases [16]. Microsatellites, also called Short Tandem Repeats (STRs), are the marker of choice for current forensic DNA profiling practices due to their high variability between individuals and their ability to be amplified using the polymerase chain reaction (PCR).

1.3 Current practices in forensic DNA profiling

1.3.1 Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) is a technique that allows for exponential copying of specific DNA sequences using only a small amount of starting material [18]. This is typically achieved through a precise three-step heating and cooling pattern (Figure 1.1). The first step involves heating the DNA to 90-95°C so that the double helix denatures, leaving two single strands. The reaction temperature is then lowered to

between 50-70°C which allows for a short sequence of DNA, called a primer, to hybridise to the complementary sequence, which flanks the target region to be amplified. The temperature is then increased to between 70-75°C, where a thermostable enzyme, Taq DNA polymerase, extends the primers by adding complementary nucleotides to make a double stranded copy of the target sequence. Each of these three steps – denaturation, primer annealing and extension of the new strand – makes up one PCR cycle. Because the PCR replicates DNA exponentially, there can potentially be up to a billion copies of the target sequence from the initial DNA template after thirty PCR cycles.

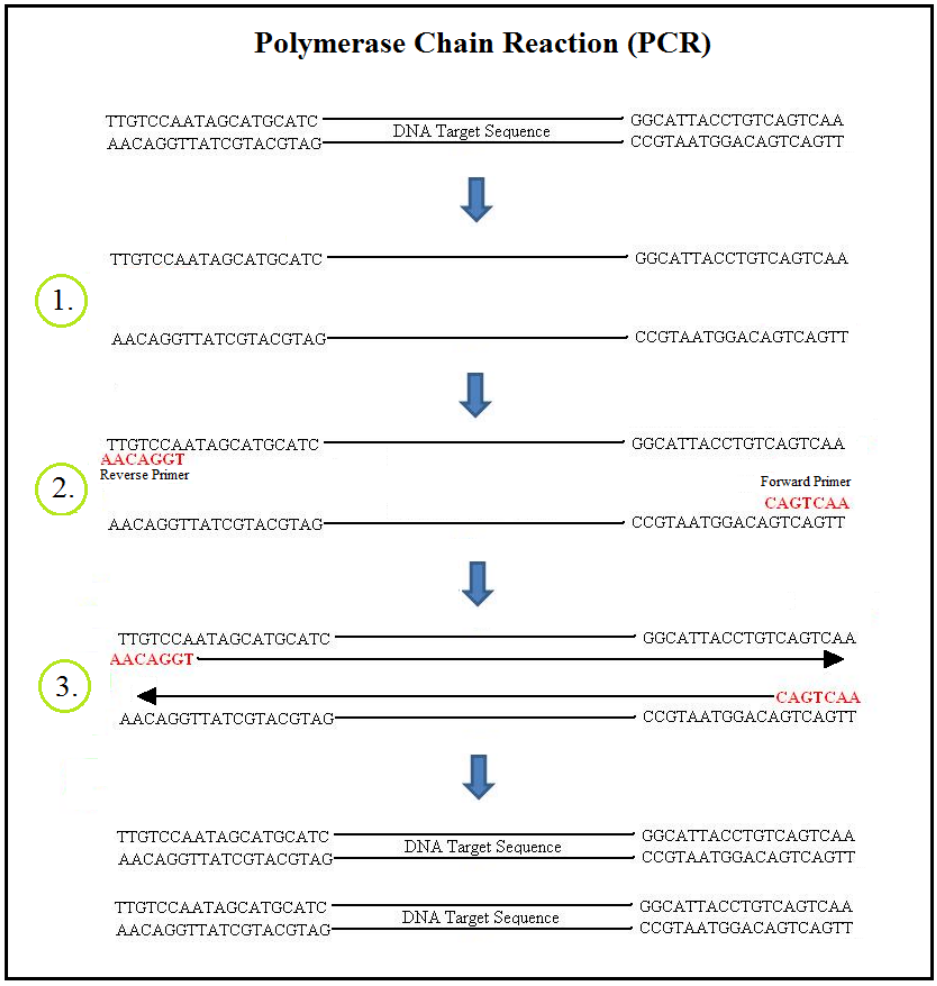

Figure 1.1 The Polymerase Chain Reaction (PCR). A precise three step heating and cooling pattern to allow for (1) denaturation of the double stranded DNA so that (2) primers can bind to the DNA flanking the target sequence. Primers are the extended by DNA polymerase (3). These steps make up one PCR cycle. There can potentially be up to one billion copies of the target sequence after 30 PCR cycles.

PCR is regarded as a reliable, reproducible and robust reaction, well suited for forensic analysis due to its ability to exponentially amplify samples with limited template quality and quantity. PCR is also suitable for multiplexing, where multiple loci can be examined in one reaction. The STR loci used for forensic human identification have core repeat sequences that are generally four bases in length, and these units are repeated a variable number of times [19]. The number of repeated segments at any particular locus can differ greatly between individuals, such that examination of multiple STR loci can identify individuals in a population with a high power of discrimination [19]. Just like the genes, every individual has two copies of each STR locus due to the maternal and paternal inheritance of chromosomes. Alleles at each locus can either have the same number of repeats, known as a homozygous locus, or a different number of repeats, known as a heterozygous locus (Figure 1.2).

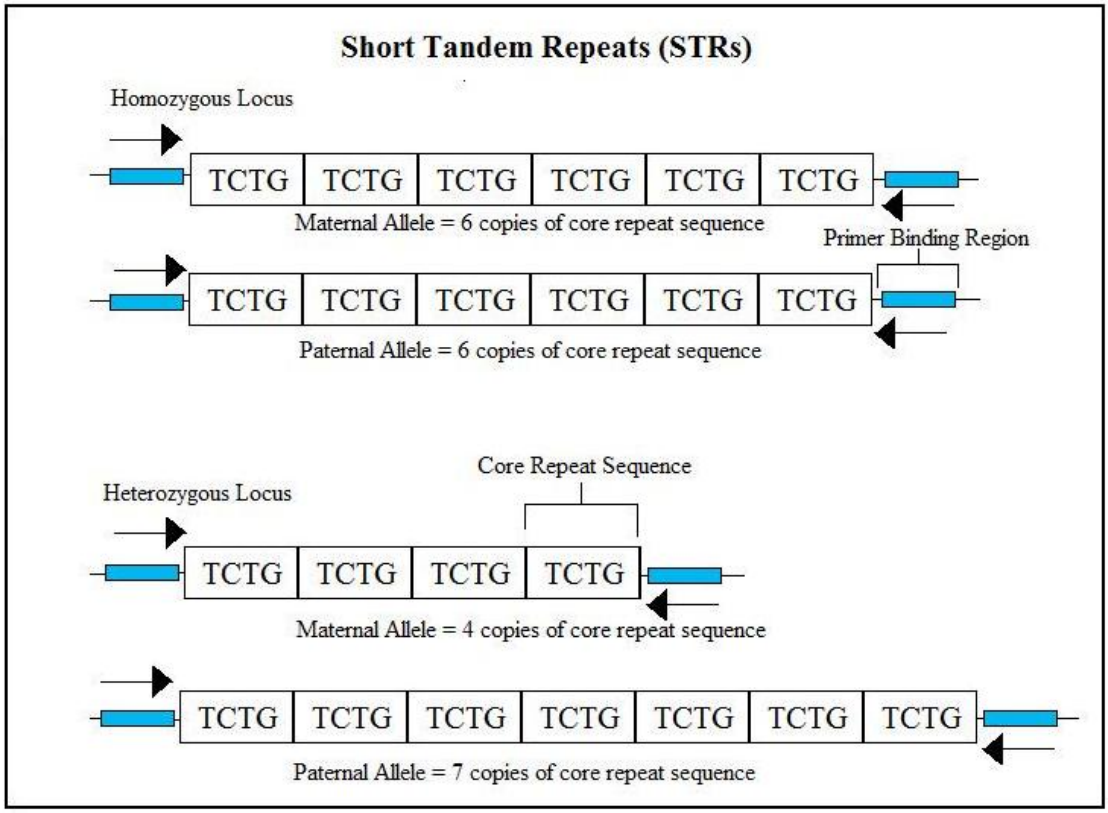

Figure 1.2 Short tandem repeat (STR) loci. The homozygous locus has two alleles with the same number of core repeat sequences. The heterozygous locus has two alleles with different numbers of core repeat sequences. Primer binding regions are constant regardless of the number of core repeat sequences.

The ability to examine multiple loci in one reaction allows for a high power of discrimination to be achieved with minimum sample consumption. PCR kits, containing polymerase, nucleotides and primers that target multiple loci, are produced commercially. The reaction conditions, namely temperatures, time at each temperature, the number of amplification cycles and the target loci vary depending on the kit used. The primers in commercial kits are tagged with one of three or four different coloured fluorescent labels. After the PCR, the fluorescently labelled product is subject to CE with laser induced fluorescence detection to obtain the DNA profile.

1.3.2 Post-PCR analysis

1.3.2.1 Capillary electrophoresis

Capillary electrophoresis is used to size separate different alleles after amplification by PCR [20]. DNA amplicons that are fluorescently labelled during PCR are injected into a capillary containing a viscous polymer. By application of an electric current, smaller, negatively charged DNA molecules will migrate more rapidly than larger fragments, such that fragments are separated by size. As the DNA fragments migrate, they are detected using a laser which excites the fluorescent label attached to the primers, causing an emission of light at a particular wavelength. The wavelength and intensity of the emitted fluorescence is detected by a Charge Coupled Device (CCD) panel. This CCD is connected to a computer system which utilises specific software to interpret the data to produce a DNA profile [20] (Figure 1.3).

The instrument software determines the size of the fragments by recording the time taken for each fragment to migrate through the capillary and comparing it to internal size standards to determine the number of repeated segments that are in each allele [20]. By analysing the size of the alleles and colour of the dye detected, the software interprets the data and provides the locus and allele information in the form of an electropherogram. The intensity of the fluorescence indicates the amount of each amplicon present in the sample, and this is represented by the height of the peak on the electropherogram, measured in arbitrary Relative Fluorescence Units (RFU).

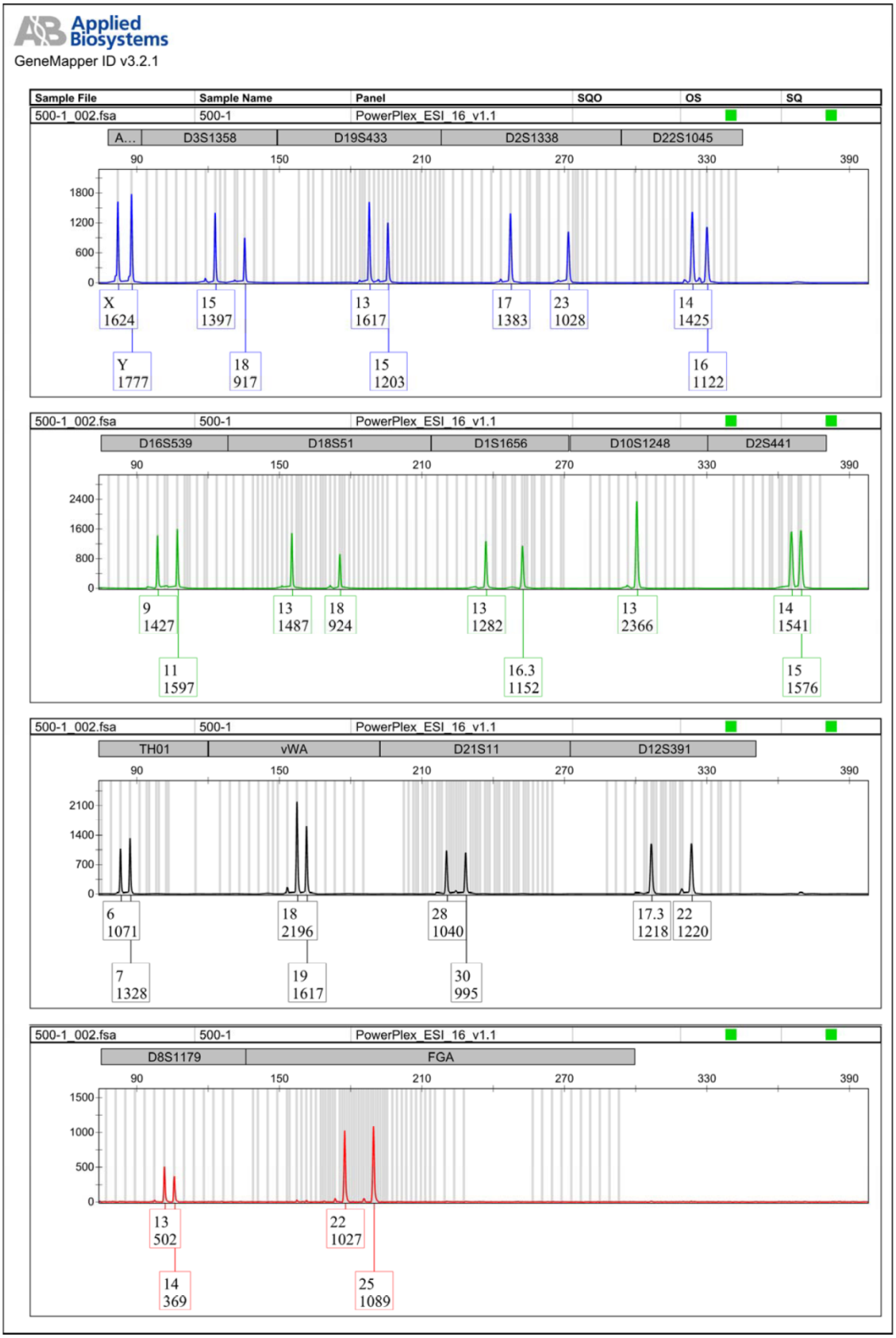

Figure 1.3 Sample electropherogram using the PowerPlex® ESI 16 system. This STR profile was obained using 500pg starting template. Boxes under the peaks display the number of core repeat units (top number) and the peak height in RFU (bottom number) for each allele.

1.3.2.2 DNA profile interpretation

When comparing unknown evidentiary DNA profiles to known DNA profiles, results can fall into one of three categories: an inclusion, where the known contributor cannot be excluded as the source of the evidentiary item; exclusion, where the person is not the source of the evidence profile; or inconclusive [21]. In order to prevent potential bias in the profile interpretation, it is imperative that evidence samples are interpreted before any individual's reference sample [22]. Evidence can be from a single source or from multiple sources. Mixture samples may contain DNA from individuals at relatively equal amounts, or may show major and minor contributors to the profile.

A number of biological artefacts can be observed in the electropherogram after PCR and CE, including stutter peaks, heterozygote peak imbalance, and near threshold peaks. These artefacts can complicate the interpretation of a profile, and therefore interpretation guidelines, such as minimum peak height thresholds, stutter ratios and minimum peak height ratios can be implemented to manage their presence in a profile. Such artefacts could also indicate the presence of a second contributor to the profile and therefore detection and interpretation thresholds and peak height ratio requirements can also assist with determining what constitutes a major and/or minor contributor in a mixed sample based [22].

1.3.2.2.1 Thresholds

In conventional DNA analysis, minimum peak heights are established to eliminate instrument noise from the profile, and peaks that fall below these thresholds are not interpreted or interpreted with caution [23]. For STR analysis, two thresholds are often set. The first, known as the analytical threshold [21] or limit of detection (LOD) [24], is generally set at around 50 RFU and reflects the sensitivity of the CE instrument. Peaks that fall below this threshold may be masked by background noise created by the instrument [24].

Consequently, this threshold helps indicate a potential allele for the profile. The second threshold is called the stochastic threshold [21] or low template DNA threshold [24]. This threshold is generally set at around 150 RFU to 200 RFU and establishes the minimum peak height for alleles such that it can be confidently concluded all alleles at a locus are present and no genetic components have failed to amplify during PCR due to a low amount of starting template, DNA degradation or PCR inhibition [22].

Peaks above the stochastic threshold are considered true alleles, such that if only one peak appears in the profile and it is above the stochastic threshold, then the locus can be designated as homozygous and included in the statistical analysis of the profile [24]. If the locus appears heterozygous with two peaks present in the profile, and both are above the stochastic threshold, then it assumed that all alleles in the sample are accounted for and this locus can also be used in the statistical analysis of the profile [24]. If a single peak appears at a locus, and this peak falls below the stochastic threshold then the locus is considered to be a potential heterozygote as one allele may have failed to amplify during the PCR process [24].

Determination of the threshold values should be based on the individual laboratory's internal validation studies [21]. If the stochastic threshold is set too high then too many true homozygote loci would be designated as potential heterozygotes, which would result in an increased number of false inclusions [24]. If it is set too low, then a locus could be erroneously designated as a homozygote, and this could result in a false exclusion, such that a suspect would fail to match the crime scene [24]. Furthermore, difficulties can arise if the peaks fall on or approach the determined threshold [4, 24]. This is particularly notable if two peaks are seen in the profile, but one is above the threshold, while the other is below the threshold [23].

1.3.3.2.2 Stutter

Stutter product occurs as a result of slipped-strand mispairing during the PCR process [25]. In this process, a region of the primer-template complex becomes unpaired during the extension stage of PCR, allowing for slippage of either the primer or template strand, with one repeat unit forming a non-base-paired loop (Figure 1.4). As a consequence, PCR product is shorter than the actual allele by one repeat unit (n-1) [25, 26]. Under conventional DNA profiling conditions, it can be difficult to determine whether a low RFU peak is a real allele from a second contributor or stutter product of an adjacent allele because stutter peaks are the same length as actual potential PCR products and are therefore seen in the same position in the electropherogram that an actual allele would be seen [25, 26].

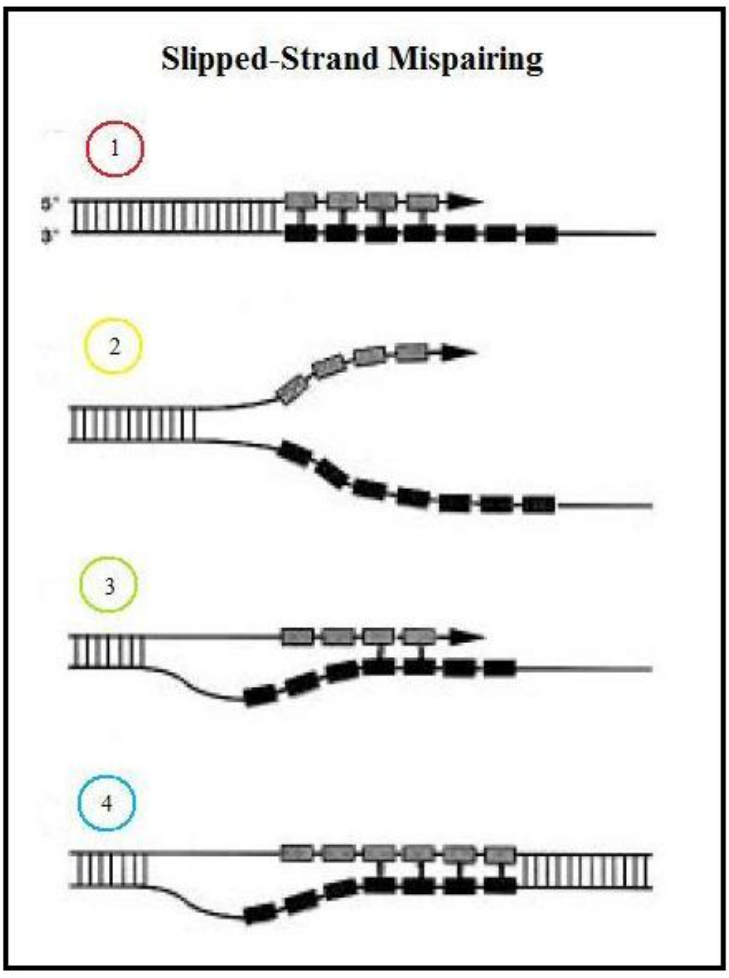

Figure 1.4 A model of Slipped-Strand Mispairing. (1) DNA polymerase extends the new DNA strand. If the DNA polymerase falls off the template strand the extending strand can break apart (2). When the two strands re-anneal the template strand loops out such that the extending strand will be misaligned with the template strand by one repeat unit (3). The newly synthesized strand is therefore one repeat unit shorter than the original template (4) [26].

While the peak height or area of the stutter peak is generally seen to be less than 10 to 15 per cent of the associated allele [26, 27] it is important for laboratories to perform internal validation studies to establish guidelines for the interpretation of potential stutter peaks as conditions can vary between laboratories depending on several factors, including the instruments and STR kits used (Figure 1.5). If the height of a peak in the stutter position is greater than the determined stutter threshold then the peak should be considered to be an actual allele from a minor contributor [27].

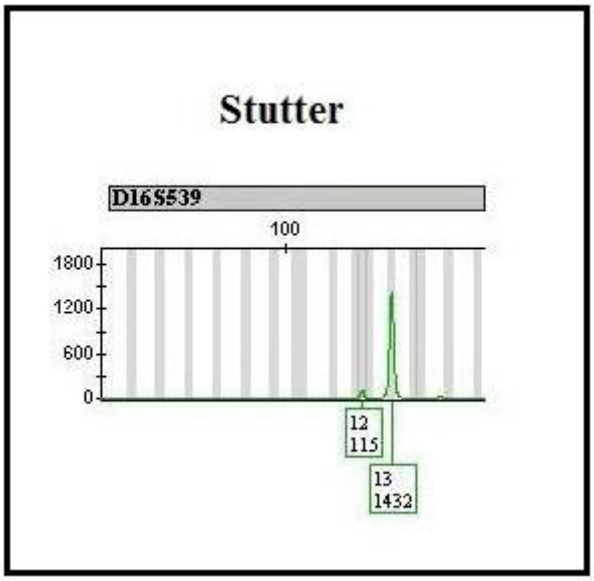

Figure 1.5 Stutter peak in the n-1 position of the 13 allele at locus D16S539. Note that the peak height of the stutter peak (115 RFU) is less than the general stutter threshold of 10% of the true allele (1432 RFU) and would generally not be considered an actually allele.

1.3.2.2.3 Heterozygote Peak Balance

Under standard PCR conditions, using the optimum amount of DNA, heterozygote peaks at a locus should be seen at relatively equal heights (Figure 1.6). If peak heights at a locus are significantly imbalanced this could be indicative of a mixture sample. Peak height ratio requirements can therefore be used to assist in determining whether a sample is from a single source or multiple sources [22]. Peak height ratios are determined by dividing the peak height in RFU of the smaller allele at a locus with the

RFU value of the larger peak, then multiplying this value by 100 to express the peak height ratio as a percentage [21]. Peak height ratio requirements should be based on empirical data, and these requirements can be separate values for each locus or one value for all loci [22]. Typically, peak height ratio threshold values range from 60-70% [22]. Samples are considered to have originated from a single source if the peak height ratios for all heterozygous loci are above the determined threshold values, and may be considered to be from more than one individual if one or more loci are below the determined peak height ratio threshold [21].

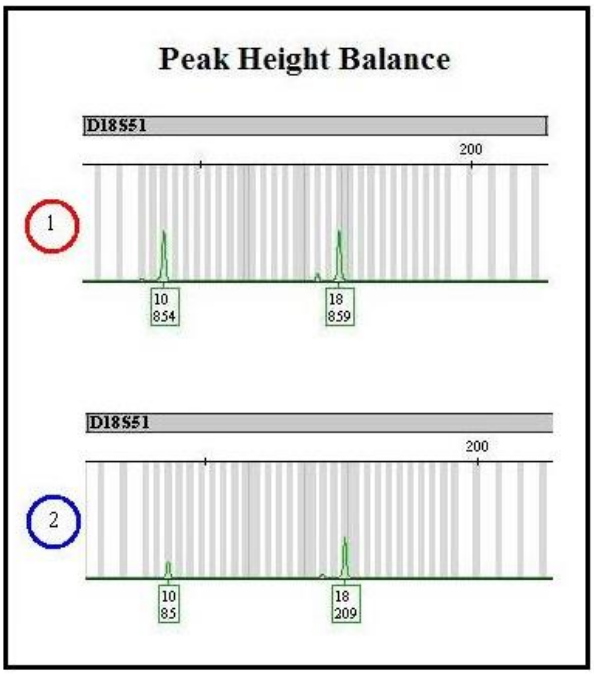

Figure 1.6 Peak height balance of alleles at heterozygous locus D18S51. Panel (1) shows alleles with similar peak heights – 854 RFU and 859 RFU – with a peak height ratio of 99.42%, indicative of a single source sample. Panel (2) shows significant peak height imbalance, with one peak at 85 RFU and the other at 209 RFU, giving a peak height ratio of 40.67% which could be indicative of a mixture sample.

1.4 Low template DNA analysis

Conventional DNA typing methods using commercial STR kits are optimised for use of approximately 200pg to 2ng of DNA to provide robust, reliable results [7-13]. However by making small adjustments to the PCR process, even smaller amounts of DNA have been profiled [14]. In 1997, using standard PCR conditions, van Oorschot & Jones reported single STR profiles from touched objects, with at least 1ng of DNA being collected from each swabbed object [28]. Later that year Findlay et al [14] reported six-STR locus profiling from a single human cell by increasing the number PCR cycles from the standard 28 to 34. In 1999 this process, known as Low Copy Number (LCN) DNA profiling, was implemented in the United Kingdom by the Forensic Science Service (FSS) [2].

1.4.1 Definition of low template DNA

Low Template DNA (LTDNA) has been variously characterised as either any technique used to increase the sensitivity of DNA profiling or using an amount of DNA below a specific level [29]. LTDNA can also be referred to as 'touch' or 'trace' DNA [23, 30]. The term LTDNA is often used interchangeably with LCN DNA, however LCN was coined by the FSS for the specific method of increasing the PCR cycle number to analyse low template DNA. There has been much debate in the literature about how to define LTDNA; however there is a general consensus for the definition proposed by Budowle et al [31] that states LTDNA is the "analysis of results below the stochastic threshold for normal interpretation". This is usually associated with samples that contain a starting template of less than 100pg of DNA, or the equivalent of approximately 17 diploid cells [2, 3, 6, 32]

1.4.2 Current methods of LTDNA analysis

The use of LCN analysis or other methods to increase sensitivity are not widespread in the international forensic community. LCN was first used by the FSS in the United Kingdom [2]. However, with the close of the FSS in 2012, there are now only two commercial providers of LTDNA analysis in the UK: Orchid Cellmark Ltd and LGC

Forensics. A 2008 review of LTDNA analysis commissioned by the UK Forensic Science Regulator reported that Orchid Cellmark and LGC Forensics use a standard 28-cycle PCR then undertake product concentration prior to optimised CE [33]. The FSS, who adopted the term LCN to describe their method, increased the number of PCR cycles from 28 to 34 using the Second Generation Multiplex Plus (SGM Plus) kit from Applied Biosystems [2]. This LCN method of increasing PCR cycle number has been used to produce DNA profiles from: single cells [14]; less than 100pg of genomic DNA [2]; touched objects, including doors, windows, clothing, and car steering wheels [34], glass, wood and metal [35] and spade and slate handles [36]; fingerprints on glass [37, 38] and paper [39, 40]; shoe insoles [41] aged and degraded bone [42] and ancient hair [43].

The LCN method has been adopted by the Environmental Science and Research (ESR) laboratory in New Zealand [6] and the Netherlands Forensic Institute [4]. A similar method has been employed by the Office of the Chief Medical Examiner (OCME) in New York State, however this laboratory only uses 31 PCR cycles, while also reducing the PCR volume, increasing the annealing time and varying the CE time and voltage [3]. Other suggested methods for LTDNA analysis include spin column purification of the PCR product [44], increasing the annealing time to 20 minutes per cycle [45], combining an increased annealing and extension time, increased cycle number and reduced primer concentration [46] or increasing the capillary injection settings [47].

1.4.3 Interpretation issues associated with LTDNA analysis

Increasing the number of PCR cycles and other LTDNA techniques as used by forensic laboratories can give improved results from both low copy number and degraded DNA samples. However, the profiles produced may be difficult to interpret and the benefits of increased sensitivity must be balanced against the reduction in quality seen in most LTDNA profiles. Common interpretation issues noted with profiles from a small starting template include stochastic sampling effects, issues with replicate analysis, difficulties in setting detection thresholds, problems with mixture analysis and

secondary transfer and the inability to determine the tissue source for many LTDNA samples.

1.4.3.1 Stochastic effects

Stochastic effects associated with LTDNA analysis have been well documented [2, 3, 5, 6, 23, 33] and are considered part of the normal results [48]. Effects such as heterozygote peak imbalance, allele and locus dropout, increased stutter height and allele drop in have been noted in LTDNA profiles since the LCN technique was first proposed by Findlay et al [14]. Such effects can lead to a reduced profile quality, making the profile problematic to interpret (Figure 1.7). Stochastic effects are particularly problematic since many LTDNA samples contain DNA from more than one individual.

1.4.3.1.1 Peak height imbalance and drop out

When sufficient starting template is present both alleles are generally amplified in equal amounts by the PCR. However, due to the kinetics of the PCR process, when sampling from a low amount of starting template, primers may not bind to both alleles at a locus in equal amounts, so that one allele may be preferentially amplified over the other [23]. This can be seen in the electropherogram as a significant height difference between the peaks of a heterozygote locus. This can cause problems in the interpretation of a profile because significant heterozygote peak height imbalance is generally a feature of a mixture in a conventional profile, while this feature is typical of LCN profiles [31].

In extreme cases of heterozygote peak imbalance, one allele may fail to amplify altogether, resulting in allele dropout [23]. This can be problematic for the interpretation of a profile as the remaining peak may be falsely designated as a homozygote, which could potentially lead to an adventitious match, or a false exclusion of the true source of the sample [23]. In some cases both alleles may fail to amplify, known as locus dropout [23]. A study by Alessandrini et al [35] found that locus drop out was mainly seen in

loci with larger amplicon sizes (300-400bp), while allele dropout is seen at approximately the same levels in all loci.

1.4.3.1.2 Stutter

Another stochastic sampling effect that can make STR profiles difficult to interpret is the increased height of stutter peaks [23]. Under standard amplification conditions, the peak height or area of the stutter peak is generally less than 10 to 15 per cent of the associated allele [26, 27]. However when examining low levels of DNA, the height of stutter peaks has been shown to increase, such that the stutter peak can be as high, or higher than the actual allele [3]. This could result in the stutter peak being mistaken for an actual allele, which could in turn result in an adventitious match or a false exclusion of the source of the sample [23].

1.4.3.1.3 Allele drop in and contamination

Due to the sensitivity of the PCR process, low levels of contaminating DNA may be amplified resulting in additional alleles seen in the profiles. This can either be allele drop in, which has been defined as occasional independent allelic events, or gross contamination, which originates from a single unknown individual that is unrelated to the crime event [30]. This contamination may be from laboratory personnel, low level DNA in reagents and consumables, sample to sample cross contamination, background contamination at a crime scene or contamination during evidence collection and handling [23]. Contamination is especially problematic when profiling from LTDNA, as methods used to increase sensitivity, such as increasing the cycle number to 34 or increasing the CE injection settings, also increase the amplification of spurious alleles that would previously not amplify to that level [35, 38, 47, 49]. In some instances the spurious alleles may be preferentially amplified over the correct alleles giving rise to a false genotype or a mixture profile that masks the true profile [35]. This can be particularly problematic if allele drop out occurs in conjunction with allele drop in, especially if the peak height of the accessory allele has reached that of the true allele, and can result in an incorrect assignment being made [35].

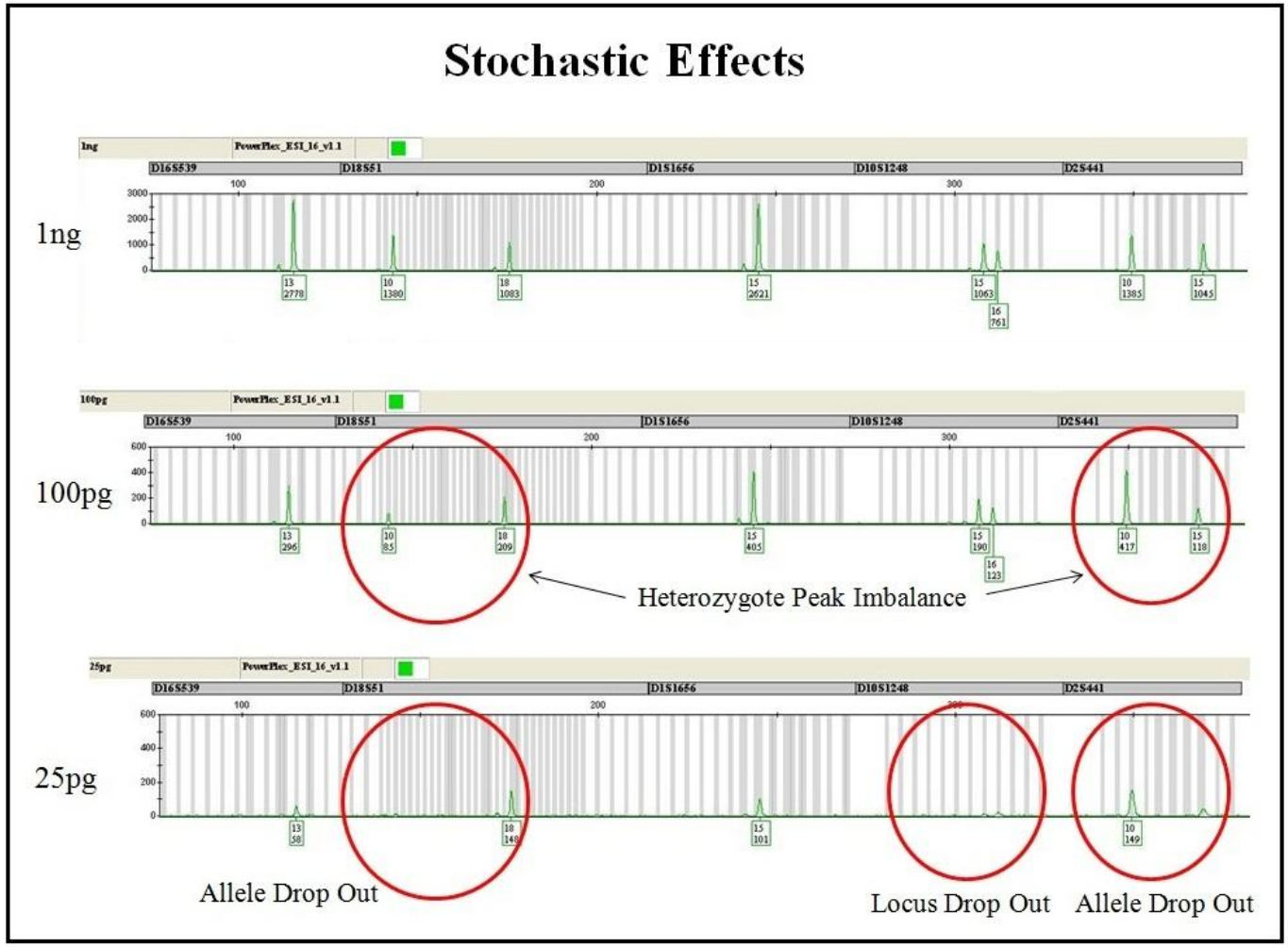

Figure 1.7 Stochastic effects observed with decreasing amounts of starting template. This image shows the green channel of a 1ng, 100pg and 25pg sample. Heterozygote peak imbalance can be seen in the 100pg profile. Allele and locus drop out are both observed in the 25pg profile. All profiles were obtained using the PowerPlex® ESI 16 Kit (Promega Corp).

1.4.3.2 Replicate analysis

Regardless of the process used to improve LTDNA profiling, stochastic effects such as allele drop out, allele drop in and heterozygote peak imbalance are expected to occur [50]. To accommodate this, current LTDNA analysis has implemented interpretation strategies to minimize the risk associated with the lack of reproducibility and stochastic effects seen in LTDNA profiles [48]. The number of attempts to genotype a low template sample is limited by the sample size [32]. Consequently when endeavouring to profile potential LTDNA samples, the entire evidence sample is generally consumed in the DNA extraction process. Therefore in order to create the impression of repeatability Gill et al [2] recommended a duplicate analysis approach, where a DNA extract is split into two or more aliquots and only alleles seen at least twice in the replicate profiles are admitted to the final DNA profile. This method, referred to as the 'Biological Model' [2], was adopted to accommodate the inherent stochastic effects of LCN DNA typing. The underlying premise of this method is that observing alleles more than once increases the confidence that an allele is a 'true' allele, assuming that contamination is not inherent in the sample [23]. This biological model is particularly useful for the elimination of non-repeating spurious alleles that appear in a profile as a result of allele drop in [2-6].

Petricevic et al [6] describes the method used by the ESR, whereby samples are amplified in duplicate and only alleles seen in both replicates included in the consensus profile. This is similar to the original FSS method, where the sample was initially split into three aliquots, but PCR and CE is only performed on two of the aliquots. The third aliquot was stored in the event that further testing becomes necessary [51]. In the FSS method, alleles at a locus had to be seen in both replicates in order to be included in the final profile [2]. The OCME in New York State and the Netherlands Forensic Institute have similar interpretation strategies to the FSS. However, in these laboratories all three aliquots are amplified [3, 4]. The NFI interpretation method involves using alleles that are detected in two or three of the replicates to form the consensus profile [4]. At the OCME, for heterozygous loci all alleles seen in at least two of the three replicates are included in the consensus profile. For a homozygous locus, the allele must appear in all three replicates [3]. Other methods of replicate analysis, such as dividing the sample

into four aliquots and including alleles seen in two of the replicates [14, 52], generating a composite profile that includes all alleles seen in the replicate profiles [53] or pooling the sample aliquots post-PCR for a single CE injection, [3, 52] have been suggested as possible alternatives to the original biological model.

A validation study of the OCME interpretation standards reported one hundred per cent correct allelic assignments over one hundred and seven non-probative case work samples and three hundred and nineteen forensic case work samples [3]. However this does not mean that a full profile was obtained for these samples, just that none of the alleles that were designated were different to reference profiles. Gill and Buckleton [54] concede that the use of replicates and the consensus strategy will not necessarily produce complete genotypes.

As stated by Budowle et al [31], reproducibility is invoked by requiring alleles to be present in multiple amplifications of the same sample. However, this generally involves diluting the samples, and diluting rather than concentrating a sample is "contrary to current effective practices" [31]. Therefore, while these replication and interpretation strategies are intended to minimize the risk involved in LTDNA analysis, Budowle, Eisenberg and van Daal [23] argue that splitting an already small sample into multiple aliquots would increase the stochastic effects seen in LTDNA profiles because fewer template molecules are subject to the PCR process in each reaction. As a consequence, differences are often noted in replicates of the same sample [4, 48, 54]. While Gill et al [2] state that the collation of results from replicates to produce a consensus profiles is a "demonstration of reproducibility", Buckleton [48] states that reproducibility is lost at low levels and it is expected that one replicate at LTDNA levels will vary from another; profiles from replicates will be broadly similar but alleles present in one replicate may be smaller or not present in another replicate. Gill and Buckleton [54] further state that reproducibility cannot be expected for replicate profiles, although results may still be reliable. Consequently, Gill and Buckleton [54] state it is not the existence of variability, but rather the magnitude and potential consequences of any variability that needs to be assessed and reported to the court, and it is then the court's responsibility to decide what weight to place on the evidence.

Budowle, Eisenberg and van Daal [23] suggest that efforts should be made to concentrate LTDNA samples rather than diluting and splitting for replicate analysis, as "common sense dictates that splitting a sample into multiple aliquots exacerbates the limitations of LCN typing". Alessandrini et al [35] state that performing replications of the PCR amplification from a LTDNA sample in order to obtain a consensus profile is difficult to apply in casework as the entire extract needs to be used to attempt positive results. However Buckleton [48] states that stochastic effects are expected even from a non-replicated sample. Gill et al [2] further state that it is still preferable to carry out duplicate analysis rather than concentrate a sample, as this did not usually increase the overall concentration of DNA above the stochastic threshold. However work from this project has shown that the most informative profile is gained by a single amplification with the entire extract [55].

Despite the issues surrounding consensus profiling, this redundancy approach is the primary method for LTDNA profile interpretation, with all laboratories conducting LTDNA analysis performing some form of replicate analysis, either duplicate or triplicate [23]. However, statistical tools are being developed that may accommodate issues such as drop in and drop out. [56-61]. If the statistical programs can incorporate these stochastic issues into the model, the evidence could be maximized by applying these tools to the DNA profile that contains the most information with the proper uncertainty/confidence associated. Such statistical tools have been implemented into some laboratories, but are generally still in the early days of development and validation and are not widespread in the forensic community.

1.4.3.3 Detection thresholds

Budowle, Eisenberg and van Daal [23] question the reliability of thresholds for LTDNA samples. Under standard PCR conditions LTDNA peak heights would normally fall below the stochastic threshold. However, efforts to increase the sensitivity of the reaction, such as increasing the PCR cycle number, can increase the peak height substantially so alleles can meet or exceed the established threshold for STR typing. Therefore peaks that do meet or exceed the threshold after manipulation do not

necessarily indicate a reliable result, since these thresholds were established using different conditions to LTDNA analysis techniques [23]. Since there is currently no purported way to establish a LTDNA typing threshold this will continue to be a weakness of the application [23].

Gill and Buckleton [30] state that thresholds cannot be set for low template samples, as the underlying model is continuous, such that there is no set point where it is absolutely certain that all events will be captured. Buckleton [48] further comments on the notion of arbitrary thresholds being imposed on continuous scales, stating, "the concept that all is good above the threshold and below it all is bad, is false, and laced with traps into which the adversarial process could lead discussion".

1.4.3.4 Mixture interpretation

Difficulties can arise in the interpretation of LTDNA profiles that appear to contain DNA from more than one source [2-4, 22, 23, 35, 49, 62-65]. Indeed, stochastic variation is most problematic for mixture samples. Issues such as ADO and peak height imbalance, combined with the possibility of allele sharing, can make determining the number or contributors or source of the contributions difficult. Many LTDNA samples are touch samples, therefore low levels of DNA from background contamination can be mixed with an evidence sample. There are currently no well developed guidelines for the analysis of such mixture profiles [22, 23]. However, many of the software programs currently being developed have incorporated statistical methodologies for mixture interpretation [57-59].

In conventional DNA profiles, features that are indicative of a mixture, such as three or more alleles at a locus or significant peak height imbalance, are typical features of an LTDNA profile [31]. In LTDNA profiles, more than two alleles at a locus could be allele drop in or a stutter peak that has been increased in height by the measures used to increase the sensitivity of the reaction [23]. Furthermore, heterozygote peak imbalance

is a common feature of profiles obtained from a low starting template amount due to preferential amplification of particular alleles [23].

LTDNA profiling is frequently more complicated when the alleles from the major contributor to a profile are above the stochastic threshold for interpretation and the alleles from the minor contributor are below the stochastic threshold [30, 49]. Furthermore, due to the increased risk of contamination and the ease at which secondary and tertiary transfer of genetic material can occur at LTDNA levels, it can be challenging to determine which, if any, of the profiles are relevant to the investigation [28, 30, 35, 39]

1.4.3.5 Secondary transfer

In one of the first studies to examine the possibility of obtaining DNA profiles from fingerprints, Van Oorschot and Jones [28] found that DNA could be transferred from objects to the hands of the next person to hold the object. Furthermore, objects that had been handled by many people all produced profiles with multiple alleles of varying intensity. Although DNA from the last holder of the object was usually present, their profile was not always the strongest one detected [28]. Balogh et al [40] found similar results in their study of DNA profiling from touched paper, such that conclusions could not be drawn on the number of donors involved in a mixture stain on the basis of allele quantity, and the strongest profile obtained was not always from the person who last touched the object. This shows that trace DNA recovered from a crime scene may not be associated with the forensic event in question. If a mixture profile is obtained it may not be possible to determine whether the mixed stain occurred from mixing of biological samples during an alleged crime, or even if DNA was deposited at the same time [30, 35]. Therefore, as stated by Alessandrini et al [35] "the possible contemporary presence of artefacts derived from amplification of LCN DNA recovered from fingerprints left on objects from multiple handlers means use of multiple allele profiles is problematic, even when proposed rules for interpretation of mixed profiles are observed".

1.4.3.6 Tissue of origin determination

Information regarding the tissue of origin for LTDNA samples can be of great importance in reconstructing the crime event in question. At present, tissue of origin determination for LTDNA samples is not possible. Most of the currently used presumptive and confirmatory tests for tissue identification are based on protein analysis, and involve enzymatic or immunological assays [17]. However, many presumptive tests have low specificity and sensitivity while many confirmatory tests are laborious and can consume much of the evidence sample [17]. Furthermore, confirmatory tests for some commonly encountered forensic tissue samples, such as saliva or vaginal secretions, are not available, [17]. Alternatives to the current protein assays, such as mRNA [66, 67] and microRNA analysis [68] or DNA methylation studies [68] are being investigated. Advantages of these methods over traditional presumptive and confirmatory test include increase specificity, faster analysis times and reduced sample consumption. Also, since these methods use PCR based techniques they could be integrated easily into the current forensic DNA workflow.

1.4.4 Challenges to LTDNA analysis

Since its introduction in 1999, there was limited challenge to the use of the LCN technique for legal purposes until the Omagh Bombing trial in 2007. In this case, the trial judge, Justice Weir, expressed concerns in relation to the handling and preservation of the DNA evidence and of the scientific validity of the 34 PCR-cycle LCN DNA profiling technique [33]. Questions were also raised about whether the LCN process had been properly validated both internally and externally, with Justice Weir stating, "The absence of an agreed protocol for the validation of scientific techniques prior to their being admitted in court is entirely unsatisfactory" [70]. This resulted in a brief suspension of LCN profiling and prompted the UK's Forensic Science Regulator to commission a review of LTDNA analysis [71].

The review acknowledged that the failure rates for LTDNA analysis were high and that LTDNA profiling had not yet achieved legal and scientific consensus, reflecting the challenging nature of analysis [33]. The review also set out twenty-one

recommendations for improvement of LTDNA analysis. These recommendations included improving education and training for police and crime scene offices on collection and storage of LTDNA samples and training and competency standards for LTDNA analysts in the laboratory. The review also recommended that all samples submitted to the criminal justice system be quantified and that national standards should be developed for LTDNA profiles in regards to extraction and quantification of DNA and interpretation of stochastic effects, mixtures and partial or contaminated profiles [33]. Despite the numerous recommendations, the review ultimately concluded that "the science supporting the delivery of Low Template DNA (LTDNA) analysis is sound" and the companies providing the service have "validated their process in accord with accepted scientific principals" [33].

Critics of the review argue that while LTDNA analysis may have investigative value the process is not ready for use as evidence in court due to the associated stochastic effects [23, 71-73]. Both Gilder et al [72] and Jamieson and Bader [73] state the conclusions of the Caddy report are inconsistent with the recommendations, and that "superficial characterisations such as 'robust' and 'fit for purpose' are a denial of the serious scientific questions that remain about the reliability and validity of LCN testing" [72]. Budowle, Eisenberg and van Daal [23] argue that because the LCN assay is not reproducible it cannot be considered robust by conventional standards. They also state that LCN is currently only appropriate for identifying missing persons and human remains and developing investigative leads, and caution should be taken if using this technique for other endeavours such as criminal proceedings [23]. Jamieson and Bader [73] suggest the lack of widespread scientific support for the LCN technique shows that it is not internationally recognised as valid or reliable. According to Gilder et al [72] this lack of consensus and the availability of alternatives to LTDNA analysis, such as miniSTRs [74-76] and mitochondrial DNA analysis, means that it is unlikely that LCN based STR testing will be embraced by crime labs in the United States. A 2004 survey of forensic service providers in Australia and New Zealand [77] revealed that most Australian laboratories did not allow variation to the standard PCR protocol, regardless of whether the sample being analysed would be considered LTDNA or conventional.

Budowle et al [31] state that before LCN typing is undertaken, limitations of analysis should be disclosed to all involved, including other laboratory personnel, supervisors, police, lawyers, the court and the public. Gill and Buckleton [54] support this call for caution and attention to potential bias, but state that "if due care is taken and the court is candidly appraised of the limitations of the technique then it is the court's purpose to weigh the strength of the evidence" and it is not the role of the scientist to act as a gatekeeper to decide whether evidence should or should not be reported.

Gill and Buckleton [54] also state that the relevance of the evidence was the true cause of confusion in the Omagh trial, not the process of achieving and interpreting the profile. The presence of trace DNA in the form of non-discrete samples such as body fluid stains or cellular debris may not indicate the originating tissue source or how it became evidential; however it is only for the scientist to consider if this DNA is from the suspect or a random man, not whether it was deposited during the commission of a crime or through other deliberate or inadvertent transfer [54]. Gilder el al [72] argue that the inability to discern the tissue source of DNA or how long DNA has been associated with an article can significantly reduce the weight that can be attached to the findings of a LTDNA profile match. In contrast Gill & Buckleton [54] state that not knowing the mode of transfer does not invalidate the profile result as long as it is correctly interpreted. However no agreement exists on how the results of LTDNA profiles should be interpreted [72, 73]. As stated by Linacre [50], "While a consensus of agreement and acceptance may be achieved, it is unlikely that a new methodology will gain absolute acceptance by all. It is also important to allow new techniques to be introduced into the criminal justice system and to encourage research". McCartney [71] warns that flawed or misinterpreted science can lead to miscarriages of justice and the court need to take precautions against admitting unreliable scientific evidence stating, "there remains an important difference between what can be reported in scientific literature and what should be used as evidence".

1.4.5 Methods to improve LTDNA analysis

Budowle & van Daal [17] suggest that rather than splitting the sample, research should concentrate on methods that reduce stochastic effects and increase the efficiency of the PCR process, such that LTDNA samples produce comparable profile results to conventional DNA samples. Methods can include making changes to the current STR practices such as: use of laser microdissection (LMD) to isolate single cells; and increasing the quantity of the sample before PCR using whole genome amplification (WGA). Examination of low template samples could also be undertaken using alternatives to STR typing such as mitochondrial DNA (mtDNA) analysis.

1.4.5.1 Changes to current short tandem repeat practices

1.4.5.1.1 Laser microdissection

Laser Microdissection (LMD), also called Laser Capture Microdissection, is a technique for isolating highly pure cell populations through the direct visualisation of cells [78-83]. Various LMD platforms exist that differ in cell separation and capture processes. Infrared capture systems involve the transfer of laser energy to a thermolabile polymer, such that the polymer melts to the selected cells to form a polymer-cell composite. Removal of the polymer from the tissue surface shears the embedded cells of interest away from the tissue section [78]. UV cutting systems involve the volatilization of cells surrounding a selected area followed by either catapulting or gravity to collect the cells of interest [78].

The ability to separate individual cells from a sample is of particular interest for forensic analysis. Mixtures of body fluids from different individuals or samples with small amounts of cells are common occurrences in forensic casework [79, 82]. LMD has been used to separate sperm cells from epithelial cells for application in sexual assault casework [79, 84, 85] as well as separating chorionic villi from the maternal component of products of conception for forensic paternity testing [80, 83]. LMD has also been used to separate blood and buccal cells from mixtures [81], non-sperm male cells from mixtures [86, 87] and the lower follicle from telogen hair [82].

STR analysis of LMD cells found that a full forensic DNA profile could be obtained from 15 to 25 epithelial cells or 30 to 50 sperm cells under standard 28-cycle PCR conditions [88-90]. Increasing the PCR cycle number to 33 or 30 cycles allowed for complete DNA profiles to be obtained from 10 or 15 epithelial cells respectively [91, 92]. Applying WGA to LMD isolated cells prior to forensic STR analysis has also been attempted [93]. With prior WGA, a complete DNA profile could be obtained from as little as 5 buccal cells [93]. However this result was not consistent, with the majority of 5 and 10 cell samples and half of the 20 cells samples showing some measure of allele drop out in the STR profile [93]. WGA of LMD isolated single or small numbers of cells has also been demonstrated in other fields such as medical and cancer genetics and these results show promise for forensic applications [94-96].

1.4.5.1.2 Whole genome amplification

WGA has been proposed as a promising method for increasing the template copy number of limited quantity DNA samples prior to traditional DNA profiling [17]. Theoretically, WGA should be capable of copying all of the DNA in a representative fashion to produce large quantities of product for standard forensic analysis [17]. Several methods of WGA have been investigated for their ability to amplify genetic material in an unbiased fashion using various amounts of starting template.

Early WGA methods used PCR to amplify the template DNA. Methods include the Primer Extension PCR (PEP) [97], Degenerate Oligonucleotide Primed PCR (DOP-PCR) [98], Tagged PCR (T-PCR) [99] and inter-Alu-PCR [100]. These techniques utilize random, partially degenerate, tagged random and Alu-based primers respectively and *Taq* DNA polymerase to amplify the genomic DNA [97-100]. Each of these methods has been used to amplify the genomic DNA from single and small numbers of sperm [98], blastomeres [101-103], and buccal cells [101], as well as various concentrations of diluted genomic DNA [99, 104, 105]. In all cases some amount of amplification failure in the downstream analysis was observed [101-105]. However, PEP and DOP were shown to produce the most complete coverage for microsatellite analysis compared to other PCR-based techniques [101]. Advancements to the PEP and

DOP-PCR methods have been introduced, with the Improved PEP (I-PEP) [105, 106], Modified I-PEP, (mI-PEP) [106] and LL-DOP-PCR (long products from low templates) [105, 107] all showing increased reaction efficiency compared to the traditional PEP and DOP-PCR methods.

Overall the PCR-based WGA methods have shown some success with downstream PCR and microsatellite analysis [97, 101, 102, 104-108]. However, these WGA procedures generally result in DNA fragments of less than 1 kb long [97, 98, 104] which could affect the success of forensic DNA profiling methods. The PCR-based techniques are also thought to produce large amounts of non-specific amplification product [104]. Additionally, PCR-based WGA has been shown to increase the incidence of -4 and +4 stutter [101, 108] which could further complicate DNA profile interpretation.

An alternative WGA method, termed Multiple Displacement Amplification (MDA), has been shown to produce higher yields [109-113] and improved genome coverage [108, 111, 113-115] compared to PCR based methods. MDA uses random hexamer primers and DNA polymerase from the bacteriophage φ29 to amplify linear DNA in an isothermal reaction, without the need for thermal cycling [116, 117]. The φ29 DNA polymerase has extremely tight binding to the DNA resulting in high processivity of the enzyme (approximately 2400 base pairs per minute) and 3' to 5' exonuclease activity for improved replication accuracy [118]. Furthermore, the use of exonuclease resistant primers allows for high DNA yields [117]. The random primers and φ29 DNA polymerase bind at multiple sites throughout the denatured DNA where new strands are synthesised. When one polymerase reaches a double strand DNA, such as that caused by adjacent φ29 DNA polymerase, it displaces the newly formed strand to continue extension. The random primers and φ29 DNA polymerase can then bind to the displaced strands where extensions of these primers form a hyperbranched DNA structure [117] (Figure 1.8).

MDA followed by STR analysis of single or small amounts of human cells has been reported, however all state that stochastic effects associated with LTDNA are still observed [119-123]. All report various levels of allele drop out, with the average number of correct loci observed per profile ranging between 53% and 80% [119, 122, 123]. Heterozygote peak imbalance was also noted in the resulting STR profiles obtained from the MDA product of single cells [119-123]. However, such phenomenon were reported to decrease when observing 2-5 cells and profiles obtained from WGA of 10 or 20 cells were comparable to profiles from conventional levels of genomic DNA [119].

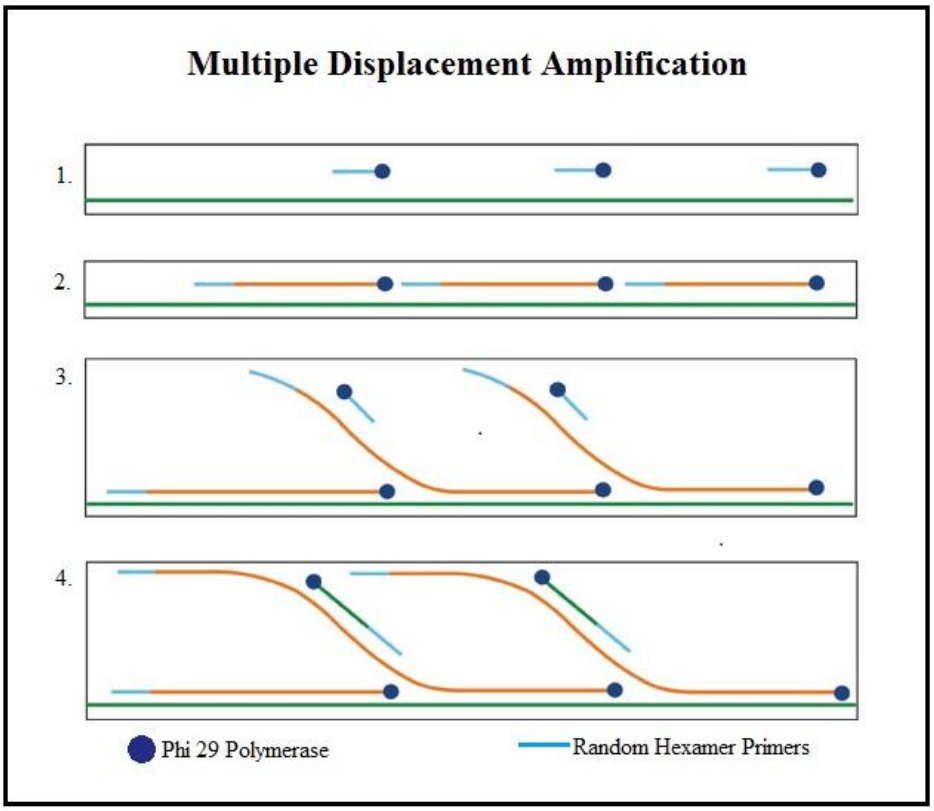

Figure 1.8 Whole genome amplification by Multiple Displacement Amplification. (1) Random hexamer primers bind to denatured DNA. (2) Phi29 polymerase extends the primers to form double stranded DNA. (3) Phi29 displaces the DNA to continue the extension of the strand, as hexamer primers bind the newly formed DNA. (4) The extension of the primers on the new strands form hyperbranched DNA. Image modified from [114].

STR profiling following MDA of DNA diluted to LTDNA amounts has also been investigated [115, 124, 125]. For each case, allele drop out was observed in the STR profile when less than 1ng [115], 500pg [125] or 250pg [126] was used as the starting template. Despite this, results showed that the profiling success of LTDNA increased with MDA, with one study reporting an average of 7 more alleles being observed in WGA samples than non WGA samples from 10pg of starting template [115]. However, none of the LTDNA samples, with or without WGA, produced STR profiles that had all alleles present [115]. Peak height imbalance, stutter and allele dropout were also observed in many samples [115, 125, 126]. A direct comparison of 28-cycle STR profiling from MDA of LTDNA obtained from fingerprints with 34-cycle STR profiling of the unamplified low level sample has been performed, with results showing that the median number of correct alleles detected from a 34 cycle PCR was 80% higher than WGA samples [126].

Methods for reducing the preferential amplification in the MDA reaction have been investigated. Such methods include the addition of molecular crowding agents to the MDA reaction [127, 128] combining MDA reactions of denatured and non-denatured DNA [129] and designing novel primers [130]. Of these methods, only the molecular crowding technique was assessed using LTDNA as the template for WGA. While the molecular crowding did improve the MDA efficiency compared to MDA without crowding, none of the MDA methods with or without crowding were able to consistently produce complete STR profiles when the starting template was less than 500pg [127].Of the other two mentioned methods, both were investigated using large amounts of DNA (more than 50ng) as the WGA template. However both reported reduced amplification bias compared to control samples [129, 130].

These studies indicate that currently, WGA using MDA can be successful when working with as little as 10 whole cells, however when observing either low levels of diluted genomic DNA or mock casework samples such as fingerprints, the stochastic effects are still amplified and the quality of the STR DNA profile can be reduced.

1.4.5.2 Alternatives to short tandem repeat analysis

1.4.5.2.1 Mitochondrial DNA analysis

The mitochondrial genome is a circular molecule consisting of 16569 base pairs. The mitochondrial DNA (mtDNA) contains a coding region, which includes sequences for two ribosomal RNAs, 22 transfer RNAs and 13 proteins, and a 1100bp non-coding or control region, also known as the Displacement loop (D-loop) [131]. This control region is of particular interest for forensic analysis as it contains two hypervariable regions, HV1 and HV2. These regions have been shown to rapidly evolve allowing for variation between individuals that can be detected through DNA sequencing for identification purposes [132]. The mitochondrial genome was originally sequenced by Anderson et al [133] and this Anderson Sequence or Cambridge Reference Sequence is, with some slight modification [134], used as a reference for which all mtDNA sequences are compared.

Mitochondrial DNA sequencing is routinely used in forensic analysis to help identify biological samples when the nuclear DNA template amount is too low or too degraded for conventional STR analysis. Each cell can potentially contain hundreds to thousands of copies of the mtDNA [135], which is significantly greater than the nuclear DNA of which there are only two copies per cell. Consequently, mtDNA assays are more sensitive, allowing for examination of samples that are typically unsuitable for traditional DNA profiling. The circular structure of mtDNA may also protect it from degradation, further increasing the likelihood of obtaining a result when autosomal STR typing is problematic [136]. Various sample types have been used for mtDNA sequencing, including bone [132, 137-141], skin [137], hair [140, 142-146], teeth [140, 141, 147], blood [139, 144, 146], fingernails [140, 146], saliva [140, 143, 148] and faeces [149], as well as numerous forensic samples such as earrings, toothbrushes, q-tips, drinking glass rims, chewing gum, razors and cigarette butts [146].

The increased sensitivity of mtDNA analysis has raised concerns regarding the ease of which contamination could occur [150, 151]. Strict anti-contamination practices must therefore be observed. Such practices can include, but are not limited to: dedicated

laboratories; use disposable gloves, caps and gowns; separation of pre- and post-PCR work areas; regular UV irradiation of equipment and; extraction and amplification of evidence prior to reference examination [151].

Another key issue that must be considered when analysing mtDNA is the inheritance pattern of the mitochondrial genome. Mitochondria are inherited from the mother as a single haplotype, with paternal mitochondria destroyed early in the embryogenesis process [152, 153]. As such any maternal relatives would, barring mutation, have the same mtDNA sequence. This has both positive and negative consequences for forensic analysis. The ability to confirm identify through any maternal relative when direct comparison cannot be made can be of great benefit, especially in cases of missing persons and mass disaster identification [132, 154-156]. However, in the identification of unknown samples collected from crime scenes, the fact that there are potentially many common HV1/HV2 types can significantly lower the mtDNA power of discrimination compared to traditional STR profiles [156]. Furthermore, since mtDNA is essentially inherited as a single locus, statistical analysis to determine the frequency of an mtDNA type in a population is often limited to the "counting method", where the number of times a particular type has been observed in various databases is presented [131, 150, 154]. Confidence intervals and likelihood ratios have also been applied to help evaluate the strength of mtDNA evidence [155].

Consideration must also be given to the fact that sequencing does not allow for the resolution of mixture samples, which is particularly disadvantageous for LTDNA samples as many contain DNA from more than one individual [157]. This can also be further complicated by the presence of heteroplasmy, a situation where more than one mtDNA type is present in a single individual [150, 151]. Both length and sequence heteroplasmy have been observed, and can occur in three ways: 1. Individuals may have more than one mtDNA type in a single tissue. 2. Individuals may show one mtDNA type in one tissue and a different type in another tissue. 3. Individuals may be heteroplasmic in one tissue sample and homoplasmic in another tissue sample [151]. Any of the described situations could complicate evidence interpretation. However, the presence of heteroplasmy does not automatically invalidate the use of mtDNA in

forensic analysis [151, 154] as long as the limitations are recognised and accounted for in evidence interpretation.

To increase the power of discrimination of mtDNA, numerous coding region SNP genotyping panels have been developed that can be used in conjunction with HV1/HV2 sequencing [158-163]. Such panels can assist in resolving common haplotypes or can be used as a screening tool to eliminate suspects prior to sequencing. Whole genome sequencing has also been suggested as a method of increasing the discriminating power of mtDNA [156, 164-167]. Initially this was performed through traditional sequencing methods [156, 164]. However, recently introduced massively parallel sequencing technologies have the potential to provide faster and less expensive methods that can achieve greater coverage in single reactions [165-167]. These methods have been shown to produce data that have a high level of consistency with Sanger-type sequencing methods [167]. However, many of the discrepancies observed were related to the analysis software's alignment algorithms, indicating room for improvement in this area [167].

1.5 Project aims

The overall aim of this project was to investigate the reliability of the current methods of LTDNA analysis and develop novel methods which could overcome the inherent issues associated with working with low amounts of DNA.

The specific aims of this thesis were:

1. To investigate whether concentrating a sample for LTDNA analysis would result in an increased quality STR profile compared with the current practice of splitting extracts into separate aliquots and constructing a consensus profile from the split sample profiles.
2. To develop a novel Pre-PCR amplification technique to increase the DNA starting template amount available for STR analysis through a non-exponential first round PCR.
3. To investigate WGA as a method for improving STR analysis of LTDNA by:

A) Examining the ability of commercial and novel WGA chemistries to amplify LTDNA for STR analysis.

B) Determining if modifications to the manufacturer's protocols could improve the MDA efficiency to result in a more complete forensic STR profile with reduced allelic imbalance.

4. To investigate the utility of LMD for routine forensic analysis, specifically:

    A) To determine if cells collected by LMD could be analysed using traditional and increased sensitivity PCR methods.

    B) To compare STR profiles from LMD cells, which therefore contain a known number of genome copies, to profiles generated from the equivalent amount of diluted genomic DNA to determine if cell samples were amplifying in the same manner as DNA dilutions.

    C) To determine if a novel WGA kit could be applied to LMD cells to produce more complete forensic STR profiles with reduced allelic imbalance.

5. To investigate the applicability of mtDNA sequencing to LTDNA samples, specifically:

    A) To determine the limit of detection of the mtDNA sequencing procedure.

    B) To examine the ability of commercial and novel WGA chemistries that target both nuclear and mtDNA to amplify LTDNA for mitochondrial control region sequencing.

    C) To examine the ability of a WGA kit that specifically targets mtDNA to amplify LTDNA for mitochondrial control region sequencing.

    D) To determine if the mtDNA specific WGA kit could be applied to LMD cells for improved mitochondrial control region sequencing.

Information gained from these studies could provide police and forensic scientists with more reliable methods for human identification from LTDNA samples. This would be particularly relevant for identification of forensic evidence samples such as touch or trace DNA, missing persons or mass disaster victims. This knowledge could also be applied to other fields such as oncogenetics, preimplantation genetic diagnosis, or ancient DNA studies where genetic analysis is often required on small cell numbers or even single cells.

CHAPTER 2

COMPARISON OF STR PROFILING FROM LOW TEMPLATE DNA EXTRACTS WITH AND WITHOUT THE CONSENSUS PROFILING METHOD

ADDENDUM

Work from this chapter has been published in *Investigative Genetics*:

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Contributions of Authors:

Kelly Grisedale:

- All molecular studies
- All data analysis
- Preparation of manuscript.

Angela van Daal:

- Participated in experimental design and coordination
- Editing the manuscript.

A response to this paper and a comment to the response were also published in *Investigative Genetics*:

- Kokshoorn B, Blankers BJ. Response to Grisedale and Van Daal: comparison of STR profiling from low template DNA extracts with and without the consensus profiling method. Investig Genet. 2013; 4:1.

- Grisedale K, van Daal A. Comment on Kokshoorn, B, and Blankers, BJ 'Response to Grisedale, KS and van Daal, A: comparison of STR profiling from low template DNA extracts with and without the consensus profiling method. Investig Genet. 2013; 4:2

Portions of this work were presented at the 23rd International Symposium on Human Identification 2012 and the 21st International Symposium of the Forensic Sciences 2012.

2.1 Introduction

Polymerase chain reaction (PCR)-based short tandem repeat (STR) analysis is considered the method of choice for forensic DNA profiling. The prominence of the technology is due to the sensitivity of detection from exponential amplification of target molecules by the PCR and the highly polymorphic nature of STRs [19]. This general method allows for small amounts of DNA, between 200pg and 2.5ng, to be analysed with commercial DNA profiling kits [7-11].

In the late 1990s, the Low Copy Number (LCN) technique was introduced to increase the sensitivity of the PCR so that substantially less DNA could be profiled [14]. With this particular process the number of PCR cycles was increased from 28 to 34 resulting in increased results from single cell DNA analysis [14]. The term LCN is often used interchangeably with LTDNA. However, in this paper, LCN will refer specifically to the technique of increasing the number of PCR cycles, whereas LTDNA will refer generically to the analysis of samples with 100pg or less starting template. Amounts less than 100pg are considered likely to produce results below the stochastic threshold for standard interpretation [31].

The LCN technique can increase the number of alleles observed in a LTDNA profile. However, interpretation difficulties can arise from the exaggerated stochastic effects associated with low levels of starting template. Such effects are well documented and include heterozygote peak imbalance, allele and locus drop out, increased stutter height and allele drop in [2-6].

To accommodate the inherent stochastic effects of LCN DNA typing, a method of replicate analysis has been adopted (referred to as the ‘Biological Model’). In this model, a sample is divided into separate aliquots, generally two or three, and a consensus profile is derived from the replicates that only includes alleles that appear in two or more of the replicates [2]. This biological method is particularly useful for the

elimination of non-repeating spurious alleles that appear in a profile as a result of allele drop in [2-6]. Other methods of replicate analysis, such as dividing the sample into four aliquots and including alleles seen in two of the replicates [4], generating a composite profile that includes all alleles seen in the replicate profiles [53] or pooling the sample aliquots post-PCR for a single CE injection, [3] have been suggested as possible alternatives to the original Biological Model.

Critics of the Biological Model suggest that splitting an already low level sample into multiple aliquots would increase the stochastic effects seen in LTDNA profiles because fewer template molecules are subject to the PCR process in each reaction. As a result, differences are more likely to be seen in replicates of the "same" sample [23]. Additionally, by creating a consensus profile, valuable information from the replicates can be lost, with one study reporting the loss of approximately one third of the alleles obtained [5]. Therefore, critics of the Biological Model advocate efforts should be made to concentrate LTDNA samples rather than diluting and splitting for replicate analysis [23].

Advocates of the Biological Model maintain that a loss of reproducibility is the normal result of LTDNA profiling and, therefore, it is not the existence of variability, but rather the magnitude and potential consequences of any variability that needs to be assessed and reported [48, 54]. It has been stated that replicate analyses are preferable to concentrating a sample as this would not usually increase the overall concentration of DNA above the 100 pg stochastic threshold, with stochastic effects still expected to occur in non-replicated samples [54]. However, little empirical evidence has been provided which shows that splitting a LTDNA extract and creating a consensus profile produces a more accurate STR profile than a concentrated LTDNA sample or vice versa.

This study aimed to investigate whether concentrating a sample for LTDNA analysis would result in an increased quality STR profile compared with the current practice of splitting extracts into separate aliquots and constructing a consensus profile from the split sample profiles. Samples with known profiles were used for all experiments. Profiles from low template samples were compared to high template reference profiles to assess profile quality. Profile quality was measured in terms of the presence of allele drop out, locus drop out and allele drop in, as well as an analysis of the peak heights and peak height ratios in profiles obtained using the different analysis methods.

2.2 Methods

2.2.1 Sample preparation

This project was approved by the Bond University Human Research Ethics Committee (BUHREC), approval number RO743. Whole blood samples were provided by five anonymous donors with informed consent. DNA was extracted from using the BioRobot EZ1® Workstation with the EZ1® DNA Blood Kits (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

The DNA extracts were quantified using a real time quantitative PCR assay. This assay amplified a 91bp fragment of 15q31, which contains the OCA2 locus. The primer sequences were 5'-GCT GCA GGA GTC AGA AGG TT-3' (forward primer) and 5'-CAT TTG GCG AGC AGA ATC C-3' (reverse primer). Primers were used at a final concentration of 200nM. This assay was performed on the Rotor-Gene™ 6000 (QIAGEN) real-time rotary analyser with 4µL of 1:100 dilution DNA extract in a 25µL reaction volume using SensiMix™ HRM Master Mix and EvaGreen dye to monitor amplification (Bioline, London, UK). PCR cycling conditions were as follows: 95ºC for 15 minutes to activate Taq DNA polymerase, then 40 cycles of 95ºC for 15 seconds, 60ºC for 10 seconds, 72ºC for 10 seconds. Genomic DNA of known concentrations (Promega Corp, Madison, WI, USA) was used to make a standard curve for quantification. Extracts were diluted to low template levels of 100pg/µl and 25pg/µl.

2.2.2 Short tandem repeat analysis

STR analysis was performed using the PowerPlex® ESI 16 Kits (Promega Corp). The manufacturer's protocol recommends 30 PCR cycles. Therefore, the samples subjected to the "Standard Cycle PCR" were amplified for 30 cycles. Samples that were analysed using the "Increased Cycle PCR" were amplified for 34 cycles. Amplification was performed in 25µl reaction volumes using a GeneAmp® PCR System 9700 (Life Technologies, Carlsbad, CA, USA).

Two series of reactions were carried out. For the first series, 100pg or 25pg of DNA templates were placed into one STR amplification reaction. Samples were amplified with 30 or 34 PCR cycles. For each template amount and cycling condition, the five donor samples were amplified in triplicate, to generate a total of 15 profiles per template amount and cycling protocol. For the second series of reactions, 15 (5 extracts amplified in triplicate) 100pg and 25pg samples were divided into 3 aliquots, so that 3 reactions containing approximately 33.3pg or approximately 8.3pg of template DNA respectively were performed for each 100pg or 25pg sample. Each 33.3pg and 8.3pg aliquot was amplified with 34 PCR cycles, giving a total of 45 of each 33.3pg and 8.3pg profiles, resulting in 15 consensus profiles for both template amounts. Reference profiles for each of the five donors were obtained using the standard cycling protocol using 500pg DNA template as recommended by the PowerPlex® ESI 16 manufacturer (Promega Corp.). Electropherograms for all samples were obtained using the 3130 Genetic Analyser (Life Technologies). For each sample, a loading cocktail of 10µl Hi-Di^{TM} Formamide (Life Technologies) and 1µl of CC5 Internal Lane Standard 500 (Promega Corp) was mixed with 1µl of amplified product and denatured for three minutes at 95°C. After cooling, samples were injected on the 3130 using a 3kv, 5-second injection as is the recommended PowerPlex® ESI 16 protocol. Data were analysed using Genemapper ID® software version 3.2.1 (Life Technologies) and PowerPlex® ESI 16 panel and bin files. A detection threshold of 50 RFU was used for analysis of all sample profiles as per Tucker et al. [12].

2.2.3 Profile interpretation

Electropherograms for all LTDNA samples were compared with 500pg control profiles (the recommended template amount for PowerPlex® ESI 16 Kits), to determine if exaggerated stochastic sampling variation was evident and to what extent. For each profile peak heights, allele drop out, locus drop out and allele drop in were noted. Peak height ratios were calculated by dividing the height of the smaller peak in a heterozygote pair by the height of the larger peak. A peak height ratio of zero was recorded if one allele in the pair failed to amplify. Peak height ratio averages were calculated in two ways. The first calculation used only the heterozygote loci that showed both alleles. The second calculation used all loci in the first calculation, as well as known heterozygote loci that had a peak height ratio of 0% due to allele drop out. While a single peak, and in effect a 0% peak height ratio, would not normally be evaluated when analysing an unknown profile, these profiles were obtained from known sources. If the peak height ratios are to be used as a measure of how well both alleles at a locus amplify during the PCR then the 0% peak height ratios are an important indicator of the efficiency of the entire reaction. If both alleles at a heterozygous locus failed to amplify, the locus was not used in calculating the peak height ratio average and median.

Locus specific stutter filters provided by the PowerPlex® ESI 16 manufacturer are as follows: 4% (THO1), 8% (D16S539), 9% (D18S1179), 10% (D2S441), 11% (FGA), 12% (D3S1358 and D10S1248), 14% (D19S433), 15% (D1S1656, vWA and D21S11), 17% (D18S51), 18% (D2S1338), 19% (D12S391) and 25% (D22S1045). Since the profiles were from known single source origins a general stutter threshold of 15% was also applied to samples that were subjected to a standard cycle PCR. Stutter has been shown to increase when measures, such as increasing the number of PCR cycles, are taken to improve the detection of low template samples [2]. To compensate for the increased stutter seen in LCN profiles, a stutter threshold of 20% was applied to samples that underwent the increased cycle PCR, based on the method of Caragine et al., who observed 97% of stutter was filtered out using a 20% filter for low template samples amplified with an increased cycle PCR and increased injection conditions [3]. If the peak height of an allele in the −4 stutter position exceeded the relevant threshold

it was designated as an allele and categorized as allele drop in. No stutter threshold was set for +4 stutter, and consequently any alleles that were present in the +4 stutter positions were designated as alleles and deemed to be allele drop in.

For each of the replicate samples in the second series of reactions, consensus profiles were constructed based on the method outlined by Caragine et al. [3], such that an allele had to be seen in at least two replicates to be included as a true allele in the composite profile.

2.3 Results

The first series of reactions, which amplified 100pg or 25pg in a single STR amplification, resulted in 15 profiles at standard cycles and 15 profiles at increased cycles for each starting template amount. Example electropherograms of the single reaction LTDNA samples and a 1ng reference sample can be seen in Figures 2.1 to 2.3 and 2.7 to 8.8. Each set of 15 profiles comprised 240 total loci. Of the total loci, 183 (approximately 76%) were heterozygous.

The second series of reactions, in which 15 100pg or 25pg samples were divided into 3 aliquots for an increased cycle amplification, produced 45 profiles, and as such 15 consensus profiles, for each template amount. Example electropherograms from 3 individual profiles used to construct a consensus profile for both template amounts can be seen in Figures 2.4 to 2.6 (100pg) and Figure 2.9 to 2.11 (25pg). All example electropherograms used DNA from the same donor, with artefacts such as allele drop out, locus drop out, increased stutter and allele drop in indicated on the profiles. An illustration on how consensus profiles were derived can be seen in Figure 2.12. Each set of 45 profiles consisted of 720 total loci, with 549 (approximately 76%) of these being heterozygous. Each set of 15 consensus profiles comprised 240 total loci, 183 of which were heterozygous.

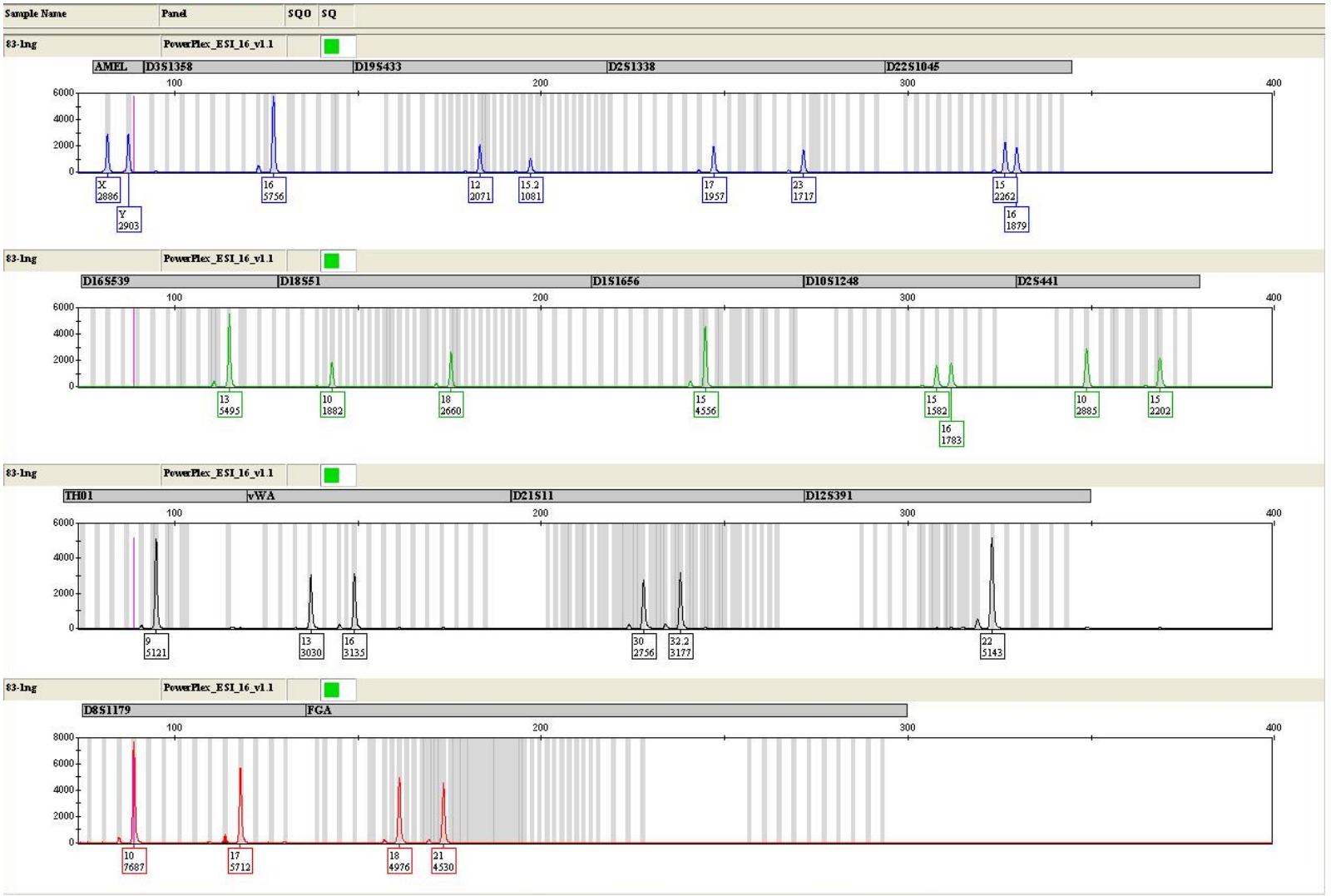

Figure 2.1 Sample electropherogram - 1ng starting template amplified with 30 PCR cycles.

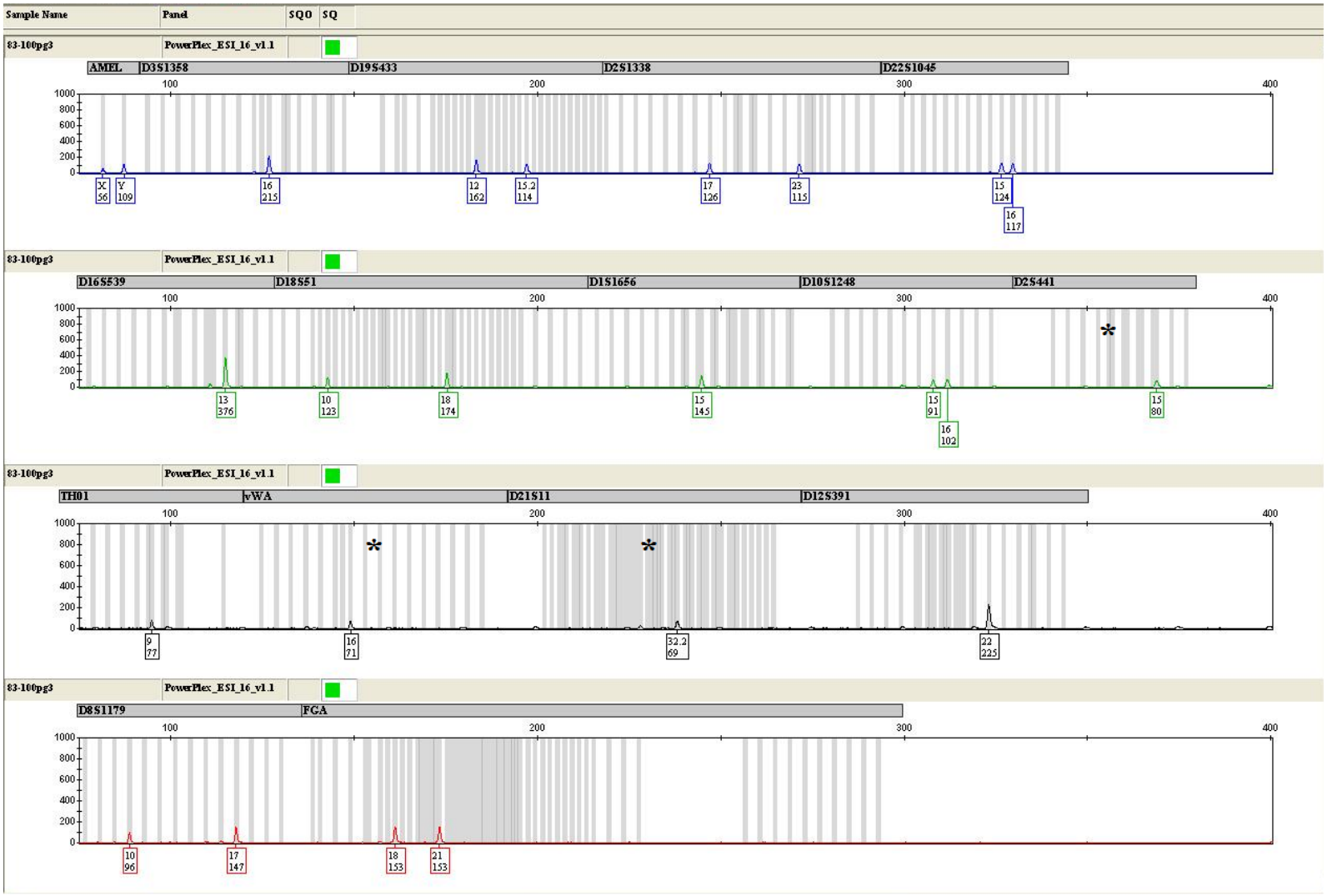

Figure 2.2 Sample electropherogram - 100pg amplified in a single reaction with 30 PCR cycles. As indicated * represents allele drop out.

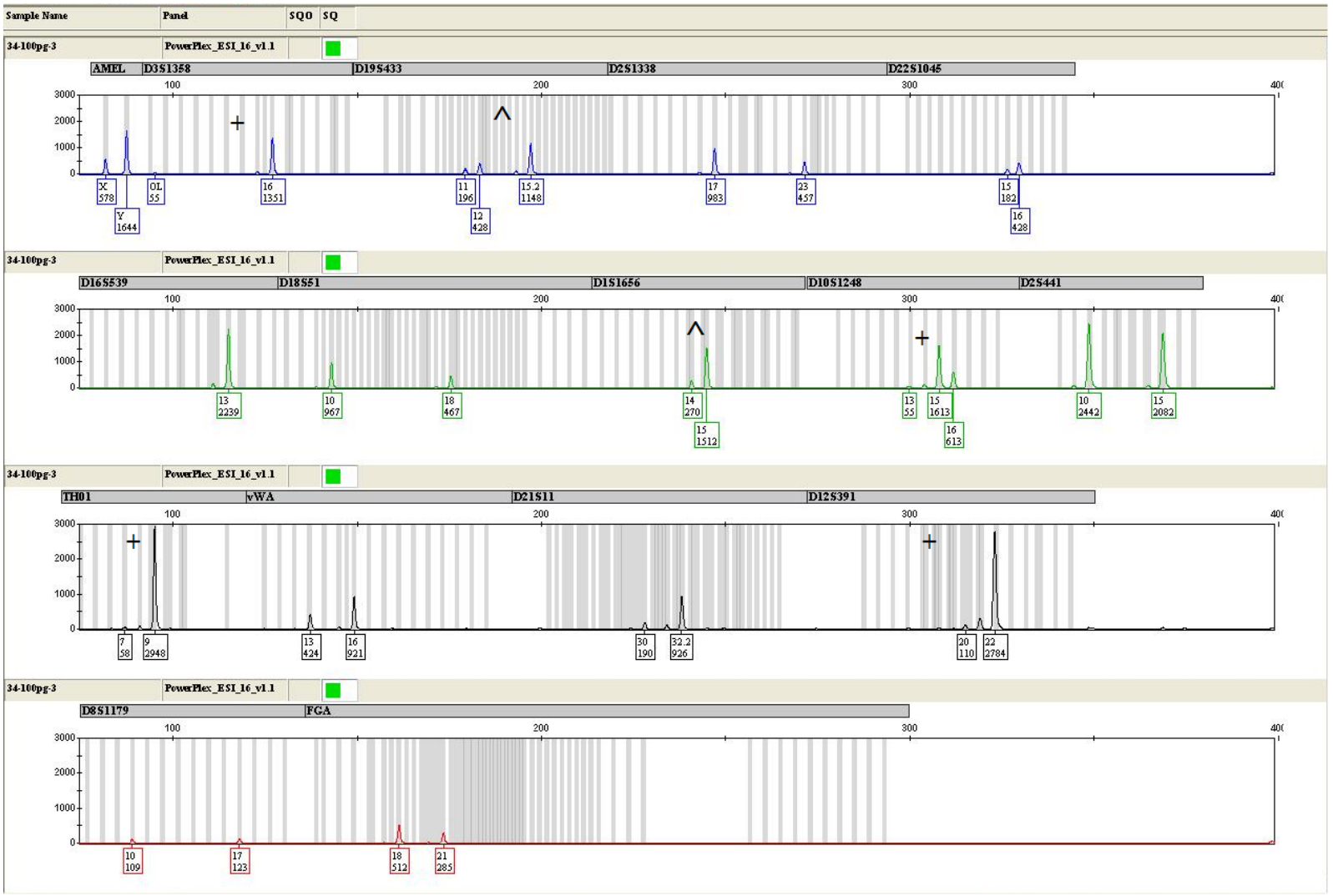

Figure 2.3 Sample electropherogram - 100pg amplified in a single reaction with 34 PCR cycles. As indicated ^ representes increased stutter and + represents allele drop in.

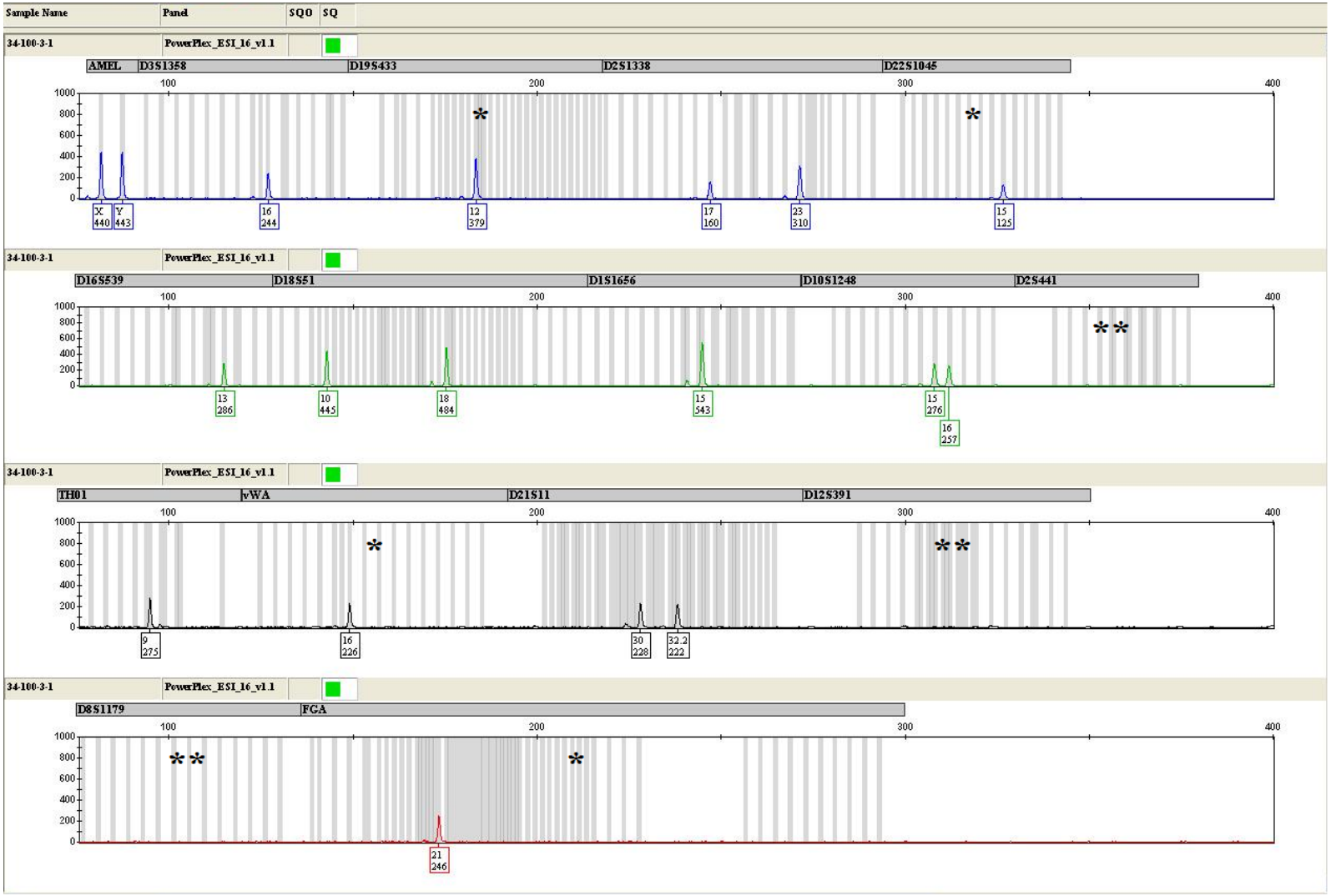

Figure 2.4 Sample electropherogram - Aliguot 1 of 100pg divided for amplification with 34 PCR cycles.
As indicated * represents allele drop out and ** represents locus drop out.

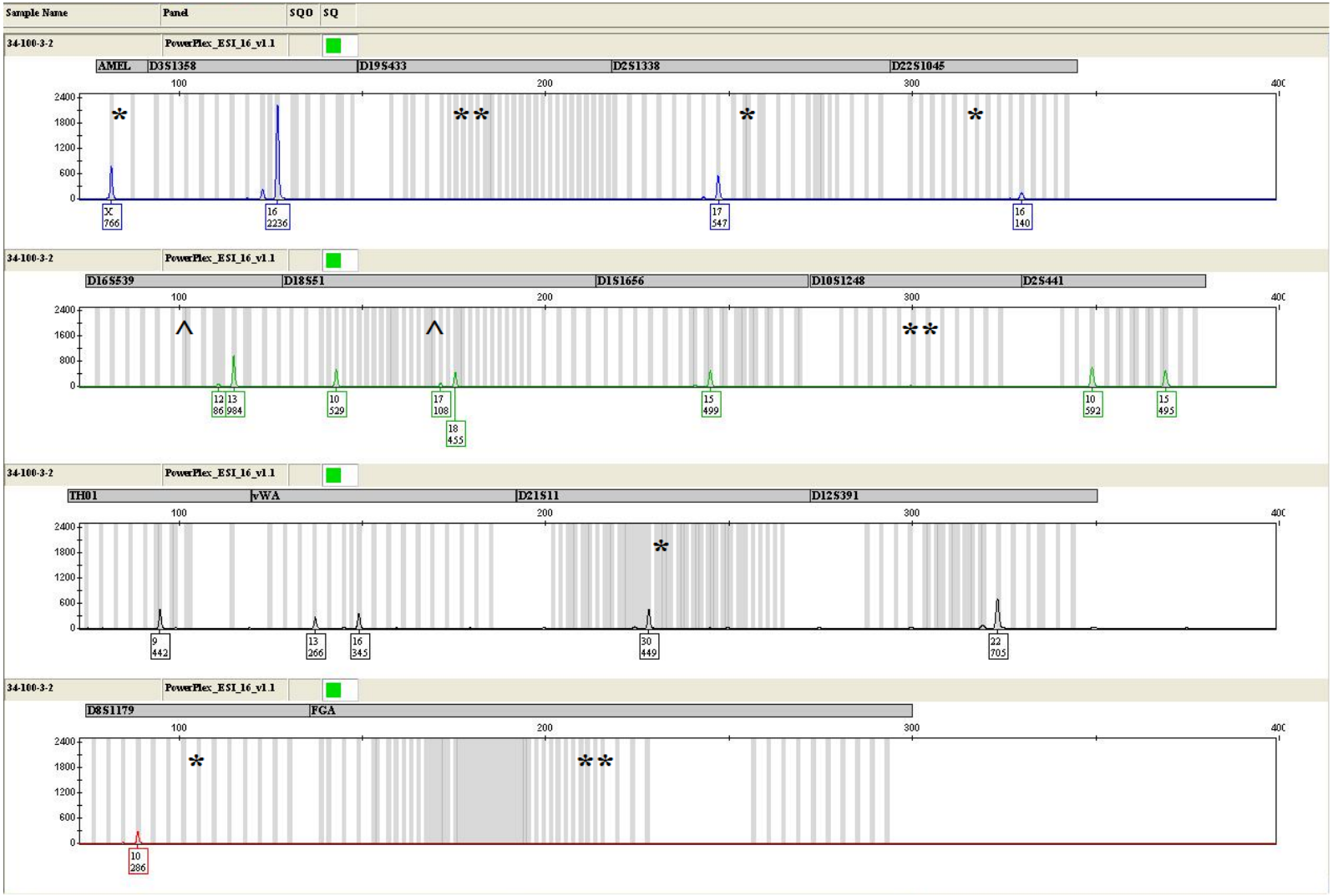

Figure 2.5 Sample electropherogram - Aliquot 2 of 100pg divided for amplification with 34 PCR cycles.
As indicated * represents allele drop out, ** represents locus drop out and ^ represents increased stutter.

Figure 2.6 Sample electropherogram - Aliquot 3 of 100pg divided for amplification with 34 PCR cycles.
As indicated * represents allele drop out and ** represents locus drop out.

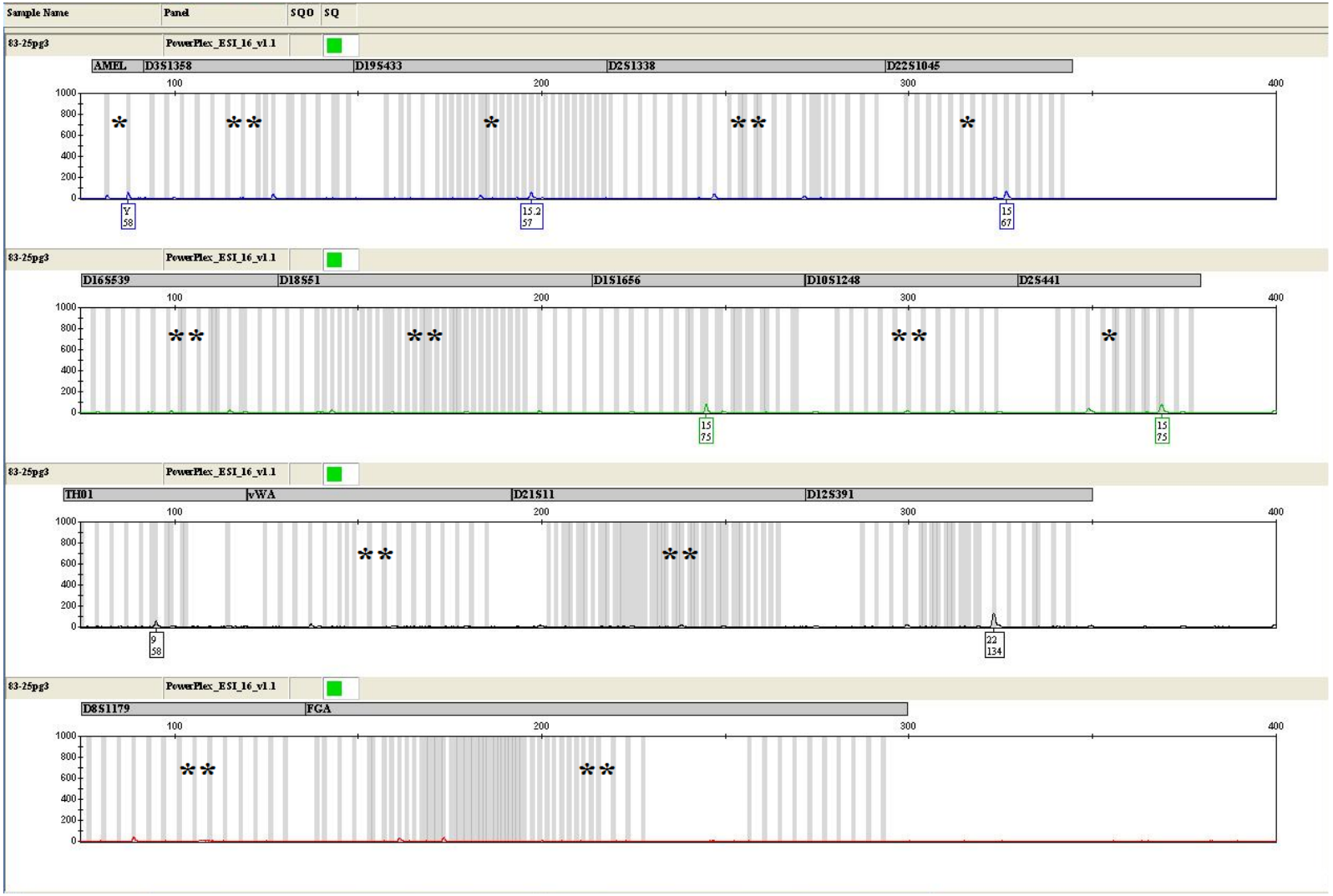

Figure 2.7 Sample electropherogram - 25pg amplified in a single reaction with 30 PCR cycles. As indicated * represents allele drop out and ** represents locus drop out.

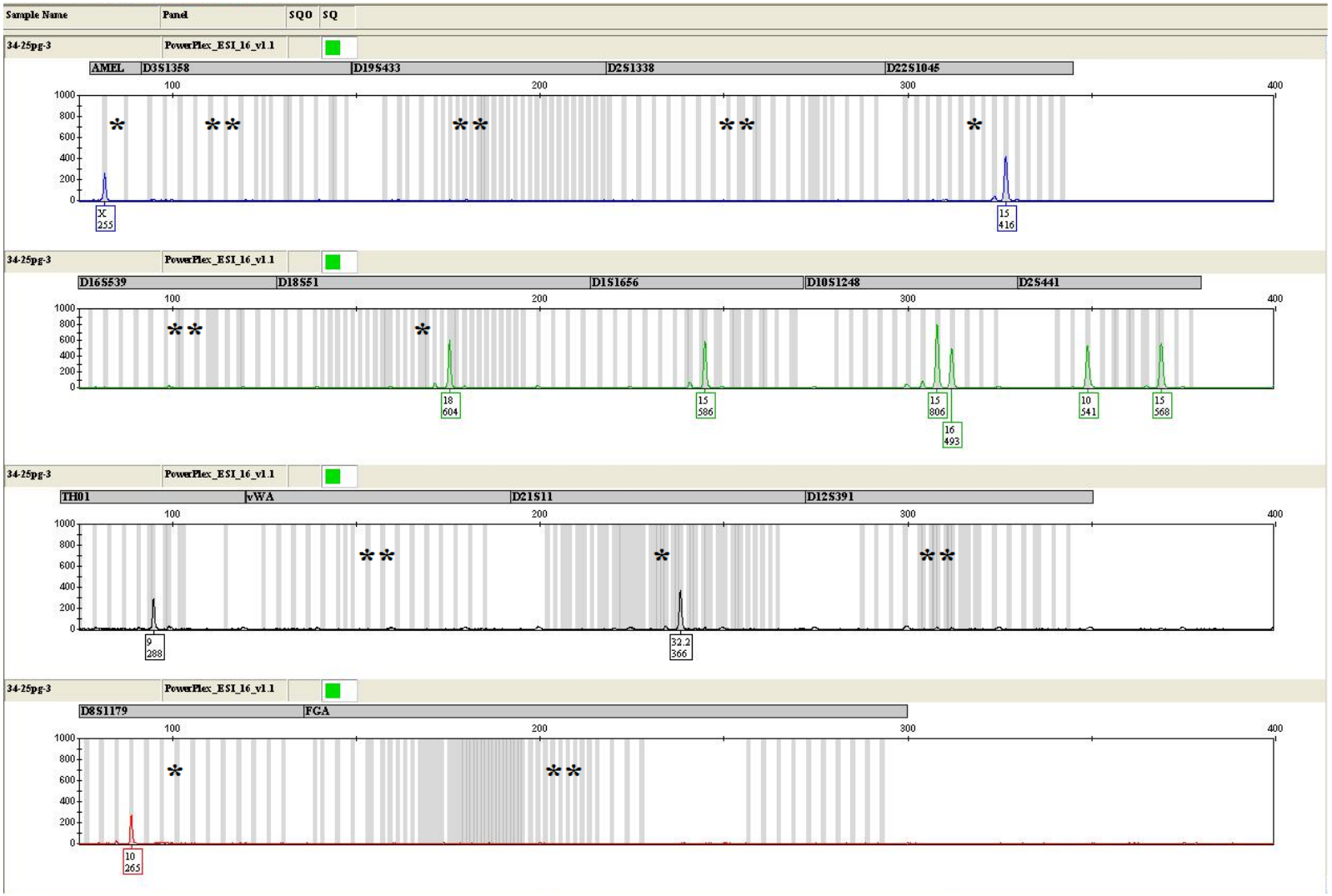

Figure 2.8 Sample electropherogram - 25pg amplified in a single reaction with 34 PCR cycles. As indicated * represents allele drop out and ** represents locus drop out.

Figure 2.9 Sample electropherogram - Aliquot 1 of 25pg divided for amplification with 34 PCR cycles.
As indicated ** represents allele drop out and ** represents locus drop out.

Figure 2.10 Sample electropherogram - Aliquot 2 of 25pg divided for amplification with 34 PCR cycles.
As indicated * represents allele drop out and ** represents locus drop out.

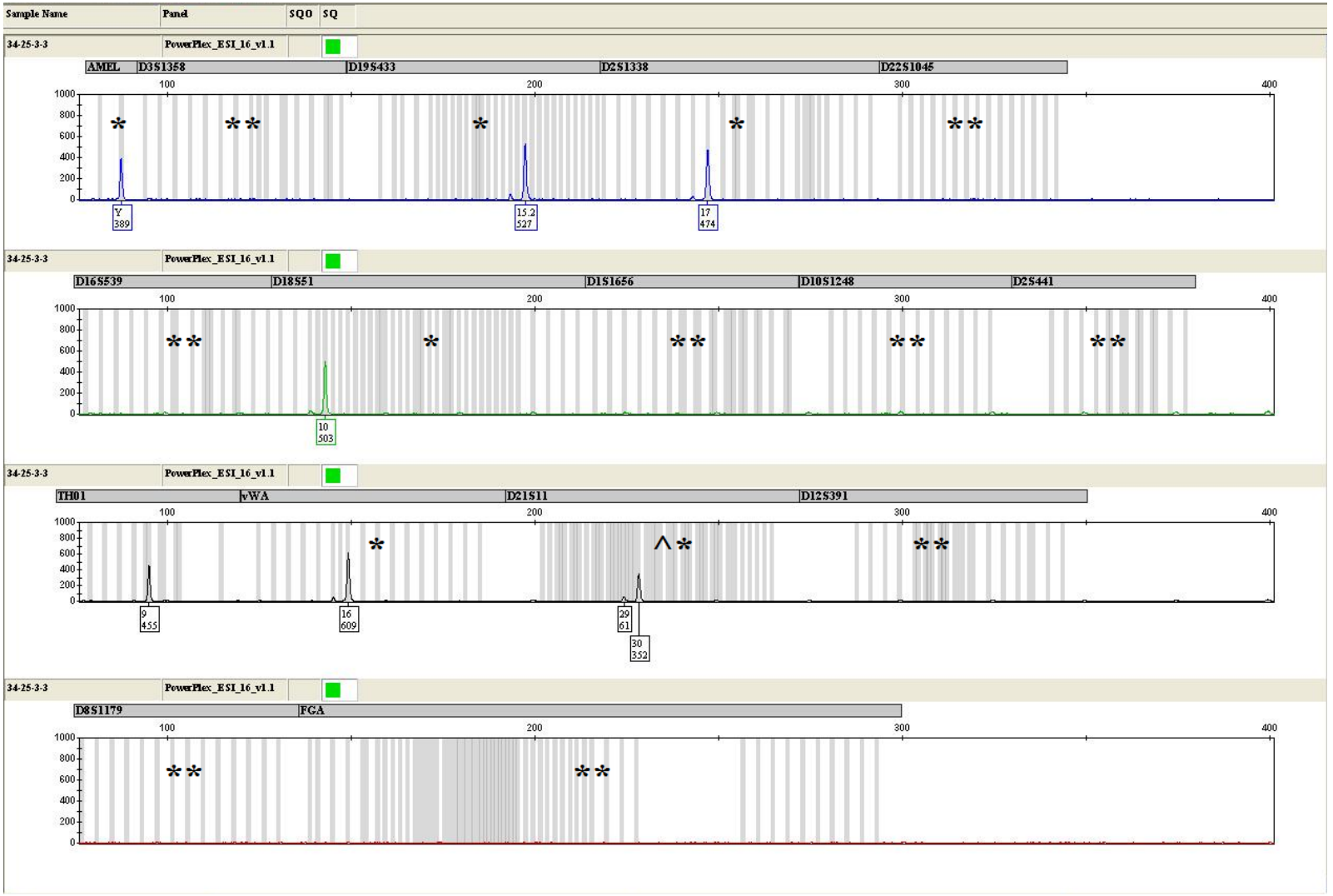

Figure 2.11 Sample electropherogram - Aliquot 3 of 25pg divided for amplification with 34 PCR cycles.
As indicated * represents allele drop out, ** represents locus drop out and ^ represents increased stutter.

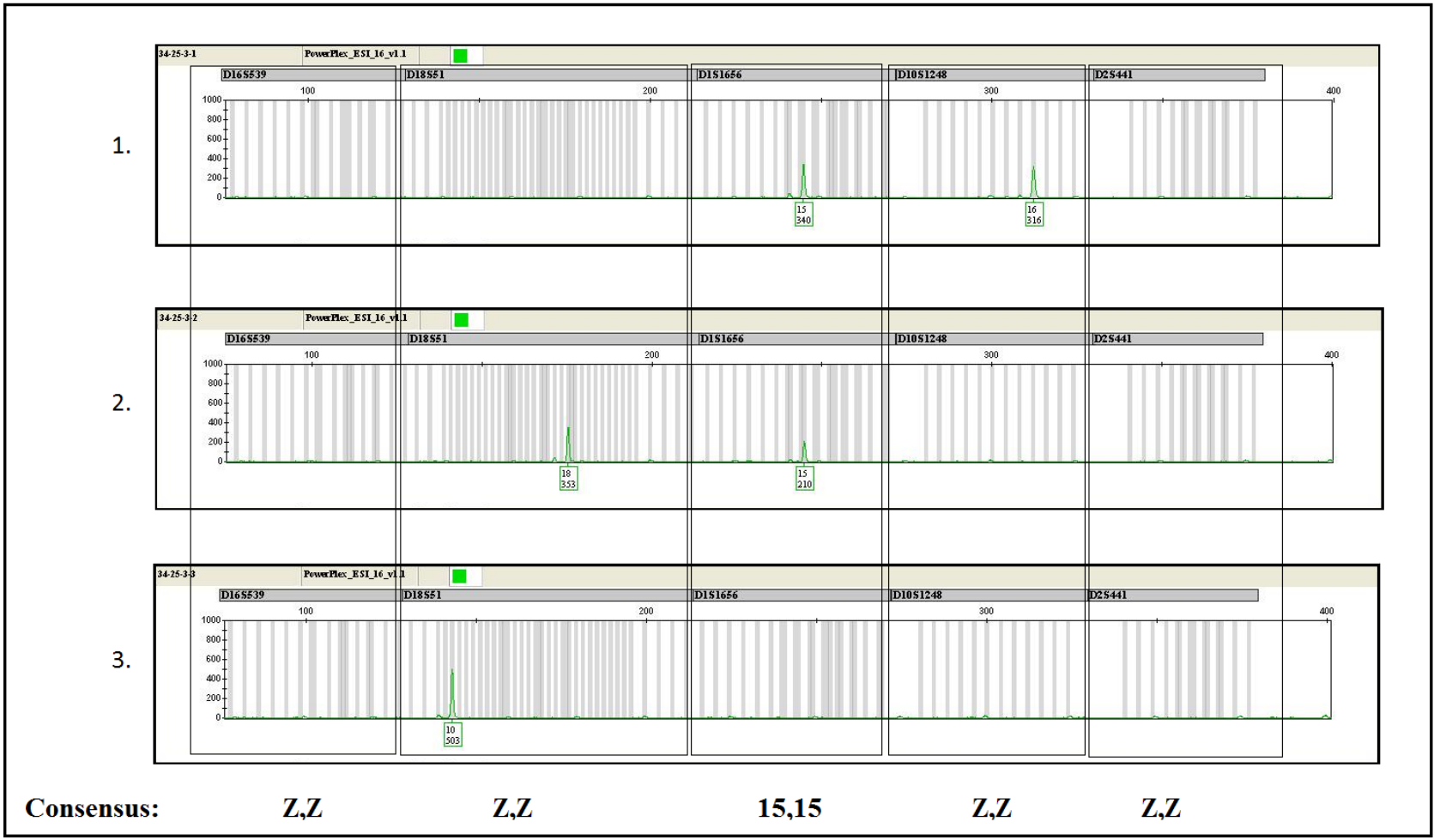

Figure 2.12 Consensus profile construction. A 25pg sample was divided into three aloquots and each amplified in a 34 cycle PCR. The green channel from each individual profile is shown. For every locus, an allele was only included in the consensus profile if it appeared in two of the three individual profiles. A 'Z' designation was given to represent potential drop out.

2.3.1 Allele Recovery

Using 100pg of starting template, allele recovery was consistent regardless of the amplification method used, with no significant differences seen between the samples amplified in a single reaction with 30 PCR cycles, samples amplified in a single reaction with 34 PCR cycles and samples divided for amplification and consensus profile construction (Figure 2.13). As shown in Figure 1, an average of 98.5% of the correct alleles were recovered in each profile using standard PCR cycling conditions, all profiles showed 100% allele recovery using a single 34 cycle PCR and an average of 96.2% of the correct alleles were seen in each consensus profile. When the starting template amount was reduced to 25pg significant differences in allele recovery were noted when comparing the 30 PCR cycle samples, which showed an average of 58.8% of the correct alleles per profile, to both the 34 PCR cycles samples amplified in a single reaction (80.5% of the correct alleles recovered per profile) and the consensus profiles (43.5% of the correct alleles recovered per profile) (Figure 1).

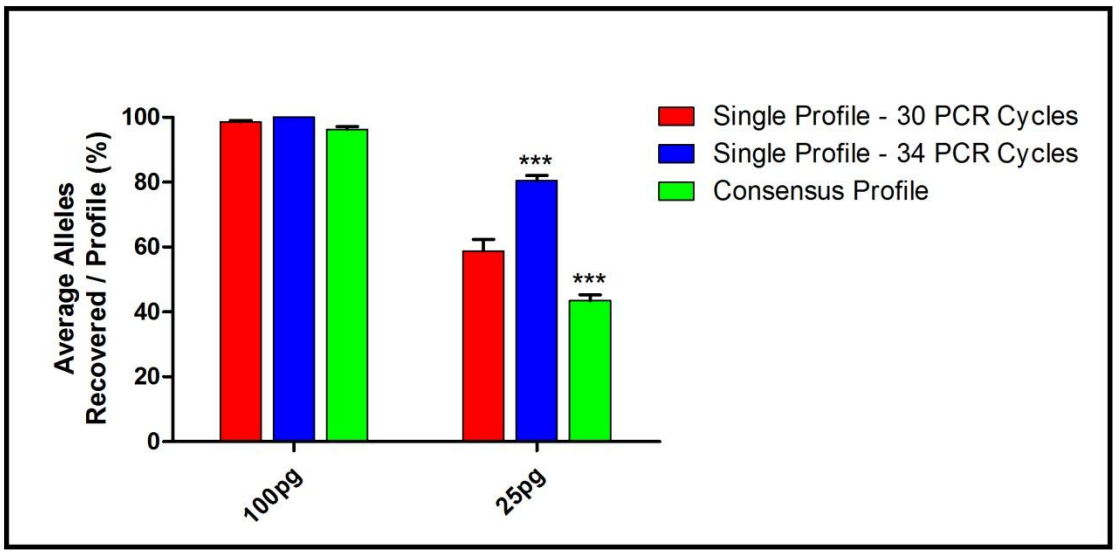

Figure 2.13 Correct alleles recovered in samples amplified using standard and increased cycle reactions. Results represent the average percentage of alleles recovered per 16-locus multiplex PCR sample. Fifteen reactions were performed for each amplification method. Error bars represent standard error of the mean. A 2-way ANOVA with Bonferroni post-tests was performed to compare allele recovery obtained using the standard 30 cycle PCR with samples amplified using the increased cycle reaction and the consensus profiling method. As indicated *** represents a p value of less than 0.001.

2.3.2 Allele drop out

The amplification of 100pg of starting template using the standard cycling protocol resulted in profiles with little observable allele drop out. Only six (3%) of the heterozygote loci showed allele drop out, with each drop out event occurring in different profiles (Table 2.1). When the number of cycles was increased to 34, allele drop out was eliminated. However, when the 100pg samples were split for amplification, the resulting consensus profiles showed an increase in allele drop out. Of the 15 consensus profiles, 16 examples of allele drop out were seen, representing 9% of the total heterozygote loci. The number of drop out alleles per consensus profile ranged from 0 to 4, with an average of 1.73 drop out events per profile.

Table 2.1 Allele drop out (ADO)

	Number of ADO	% Heterozygote Loci with ADO
100pg Starting Template		
30 PCR Cycle Amplification		
Non-split Samples (n[a] = 183)	6	3%
34 PCR Cycle Amplification		
Non-split Samples (n = 183)	0	0%
Split Samples (n = 549)	114	21%
Consensus Profiles (n = 183)	16	9%
25pg Starting Template		
30 PCR Cycle Amplification		
Non-split Samples (n = 183)	80	44%
34 PCR Cycle Amplification		
Non-split Samples (n = 183)	61	33%
Split Samples (n = 549)	250	46%
Consensus Profiles (n = 183)	92	50 %

[a] n is the total number of heterozygote loci

Allele drop out significantly increased as the amount of starting template was reduced. Using the standard cycle protocol, amplification of 25pg of starting template resulted in 80 occurrences of allele drop out over 15 profiles, representing 44% of the total heterozygous loci (Table 2.1). The number of drop out alleles per profile ranged between 3 and 8, with an average of 5.4 allele drop out events per profile. When the samples were subjected to an increased cycle PCR, the percentage of allele drop out was

reduced to 33%, with 61 examples of allele drop out over 15 profiles. The number of drop out alleles in each profile obtained using the increased cycling method ranged between 2 and 7, with an average of 4.07 per profile. However, when 25pg of starting template was split for amplification the resulting consensus profiles showed an increase in allele drop out, with 92 cases over the 15 consensus profiles, which corresponds to 50% of the total heterozygous loci. The number of drop out alleles per profile ranged between 2 and 10, with an average of 7.67 drop out events in each profile.

2.3.3 Locus drop out

For the purpose of this study, and in all subsequent chapters, locus drop out was defined as the single allele from a homozygous locus, or both alleles from a heterozygous locus missing from the profile. In the latter case, both missing alleles were not each counted individually as allele drop out. Locus drop out was not seen in any of the profiles obtained from 100pg starting template, regardless of whether the sample was amplified using the standard or increased cycle PCR (Table 2.2). When the 100pg samples were divided into three 33pg aliquots and used to construct a consensus profile, the individual profiles did show some locus drop out, with 12 instances seen across the 720 loci. The consensus profiles derived from the aliquots were complete and correct since locus drop out did not occur at the same locus more than once in any set of three replicate profiles.

Locus drop out was much more evident in the 25pg samples (Table 2.2). Under standard cycling conditions, 51 examples of locus drop out were recorded over the 240 total loci (21%). Between 0 and 7 loci dropped out per sample, with an average locus drop out of 3.4 per sample. This drop out was reduced when the number of PCR cycles was increased, with only 13 (5%) of the total loci dropping out. Under the increased cycle amplification condition, the number of locus drop out events per sample ranged between none and two, with an average of less than one drop out locus per profile. However, when the samples were split and a consensus profile was derived, locus drop out increased, with 79 instances seen in the 15 consensus profiles, representing 33% of the total loci. The number of drop out loci per sample ranged from 3 to 10, with an average of 5.27 loci dropping out in each consensus profile.

Table 2.2 Locus drop out (LDO)

	Number of LDO	% Loci with LDO
100pg Starting Template		
30 PCR Cycle Amplification		
Non-split Samples (n[a] = 240)	0	0%
34 PCR Cycle Amplification		
Non-split Samples (n = 240)	0	0%
Split Samples (n = 720)	12	2%
Consensus Profiles (n = 240)	0	0%
25pg Starting Template		
30 PCR Cycle Amplification		
Non-split Samples (n = 240)	51	21%
34 PCR Cycle Amplification		
Non-split Samples (n = 240)	13	5%
Split Samples (n = 720)	245	34%
Consensus Profiles (n = 240)	79	33%

[a] n is the total number of loci

2.3.4 Allele drop in

Allele drop in was minimal under standard cycling conditions, with only two additional alleles seen across all 100pg sample profiles, one of which was seen in the −4 stutter position and the other seen in the +4 stutter position (Table 2.3). Drop in increased when the samples were amplified with the increased cycle PCR, with 32 additional alleles seen in the resulting 15 profiles. The number of drop in alleles per sample ranged between 0 and 4, with an average of 2.13 additional alleles seen in each sample. Allele drop in also occurred in the profiles of the split samples, with a total of 32 additional alleles seen in the 45 split sample profiles. However, the consensus method requirement for an allele to be seen twice effectively counteracted this drop in, so that no additional alleles were seen in the 15 consensus profiles.

A similar pattern was observed in the 25pg sample profiles. No additional alleles were seen in the standard cycle PCR profiles, but 6 drop in alleles were noted in the 15 increased cycle PCR profiles. When the samples were divided into 3 aliquots for amplification, 15 additional alleles were seen in the 45 split sample profiles. However,

again, the consensus method eliminated this drop in, so that no additional alleles were seen in the consensus profiles.

Table 2.3 Allele drop in (ADI)

	Allele Drop In Placement			Number of ADI	% Loci with ADI
	Minus 4	**Plus 4**	**Random**		
100pg Starting Template					
30 PCR Cycle Amplification					
Non-split Samples (n[a] = 240)	1	1	0	2	0.3%
34 PCR Cycle Amplification					
Non-split Samples (n = 240)	2	10	20	32	13%
Split Samples (n = 720)	11	11	10	32	4%
Consensus Profiles (n = 240)	0	0	0	0	0%
25pg Starting Template					
30 PCR Cycle Amplification					
Non-split Samples (n = 240)	0	0	0	0	0%
34 PCR Cycle Amplification					
Non-split Samples (n = 240)	2	1	3	6	3%
Split Samples (n = 720)	10	1	4	15	2%
Consensus Profiles (n = 240)	0	0	0	0	0%
Total (n = 2,400)	**26 (30%)**	**24 (27%)**	**37 (43%)**	**87**	**4%**

[a] n is the total number of loci

Of the 87 additional alleles observed across all profiles, 26 were seen in the '-4' stutter position. Alleles in this position were only counted as drop in if their peak height exceeded the nominated stutter ratio filters (locus specific stutter filters followed by a manual examination using a 15% filter for samples amplified with the standard number of cycles and 20% for samples amplified with an increased cycle PCR). Indeed, 140 additional peaks were actually seen in −4 stutter positions; however, 114 were removed from the final profiles by the stutter filters. Twenty-four additional alleles were observed in '+4' stutter positions. While 23 of the 24 additional alleles in +4 positions had peak heights less than 20% of the true allele, a filter was not set for +4 stutter; therefore, additional alleles in this position were counted as drop in. The remaining 37 additional alleles were placed throughout the profiles.

2.3.5 Peak heights and peak height ratios

For the 100pg samples amplified with 30 PCR cycles, the height of homozygous peaks ranged from 185 to 847 RFU, with an average peak height of 520 RFU. The height of heterozygous alleles ranged between 55 and 725 RFU, with an average of 261 RFU (Table 2.4). The peak height ratio range for heterozygote loci was 16% to 99% with a peak height ratio average of 69%. When taking into account the heterozygote loci that had a peak height ratio of 0% due to allele drop out, the average was reduced to 67% (Table 2.5).

The peak heights increased when the number of PCR cycles was increased to 34. For homozygous alleles, the peak heights ranged between 622 and 7,609 RFU with an average height of 4,129 RFU. The peak height range for alleles at heterozygous loci was 139 to 5,805 RFU with an average height of 1,925 RFU (Table 2.4). However, increasing the number of PCR cycles resulted in a slightly reduced peak height ratio average of 65%, with a peak height ratio range of 6% to 100% (Table 2.5). Allele drop out was not seen in any of the 100pg increased cycle profiles; therefore, only one calculation was performed.

The 100pg samples that were split for amplification and were subject to 34 PCR cycles displayed peak heights higher than those subjected to the 30-cycle amplification, presumably because the increased number of cycles compensates for the decreased template amount. The heights of homozygous peaks ranged from 166 to 6,131 RFU, with an average height of 1,610 RFU. The heterozygous loci showed a peak height range of 56 to 4,123 RFU, with an average peak height of 763 RFU (Table 2.4). The peak height ratio range for heterozygous loci showing both alleles was 8% to 99%, with an average of 57%. Inclusion of the 114 heterozygote loci that had a 0% peak height ratio, the average was reduced to 45% (Table 2.5).

Table 2.4 Peak heights

	Homozygous Peaks (RFU)				Heterozygous Peaks (RFU)			
	n	**Range**	**Mean**	**Std Dev.**	**n**	**Range**	**Mean**	**Std Dev.**
100 pg Starting Template								
30 PCR Cycle Amplification								
Non-split Samples	57	185 to 847	520	170	360	55 to 725	261	116
34 PCR Cycle Amplification								
Non-split Samples	57	662 to 7609	4129	1767	366	139 to 5805	1925	1233
Split Samples	168	166 to 6131	1610	1090	949	51 to 4975	763	638
25 pg Starting Template								
30 PCR Cycle Amplification								
Non-split Samples	49	51 to 337	149	73	200	50 to 360	110	56
34 PCR Cycle Amplification								
Non-split Samples	53	212 to 3475	1259	805	287	58 to 3500	708	602
Split Samples	122	59 to 2144	541	369	458	53 to 2074	386	260

Table 2.5 Peak height ratios (PHR)

	ADO (0%) Included In the PHR				ADO (0%) Not Included In the			
	n[a]	**Mean PHR**	**Std.**	**Median**	**n[b]**	**Mean PHR**	**Std.**	**Median**
100 pg Starting Template								
30 PCR Cycle Amplification								
Non-split Samples	183	67%	23%	70%	177	69%	20%	71%
34 PCR Cycle Amplification								
Non-split Samples	183	65%	22%	69%	183	65%	22%	69%
Split Samples	540	45%	32%	47%	426	57%	24%	55%
25 pg Starting Template								
30 PCR Cycle Amplification								
Non-split Samples	140	29%	36%	0%	60	68%	18%	65%
34 PCR Cycle Amplification								
Non-split Samples	174	36%	32%	38%	113	56%	22%	52%
Split Samples	353	19%	32%	0%	103	64%	24%	60%

[a] n is the number of heterozygote loci with at least one allele present [b] n is the number of heterozygote loci with both alleles present

Amplification of 25pg starting template resulted in a peak height reduction compared with the 100pg samples. Under standard PCR cycling conditions, the heights of homozygous peaks ranged between 51 and 337 RFU, with an average of 149 RFU. For heterozygous loci, the peak height range was 50 to 360 RFU, with an average height of 190 RFU (Table 2.4). The peak height ratios range for the heterozygous loci was 35% to 99% with an average 68%. When all heterozygous loci that showed at least one allele were included in the calculation, the average peak height ratio was reduced to 29% (Table 2.5).

As with the 100pg samples, by increasing the number of PCR cycles the average peak height for the 25pg samples also increased. For homozygous alleles, the average peak height was 1,259 RFU, with a range of 212 to 3,475 RFU. The height of heterozygous alleles ranged between 58 and 3,500 RFU, with an average height of 708 RFU (Table 2.4). Considering only the heterozygous loci that showed both alleles, the peak height ratio range was 10% to 99%, resulting in a peak height ratio average of 56%, a reduction compared to the average of the standard cycle samples. However, when the heterozygous loci that had a peak height ratio of 0% were included in the average calculation, the peak height ratio average was higher compared to the standard cycle profiles at 36%, due to the reduction in allele drop out (Table 2.5).

For the 25pg samples split for amplification, the heights of the alleles were increased compared with the standard cycle amplification samples due to the increased number of PCR cycles utilized. For homozygous alleles, the peak heights ranged from 59 to 2,144 RFU; however, the average height was only 541 RFU. The range for heterozygous allele heights was similar at 53 to 2,077 RFU, with an average height of 386 RFU (Table 2.4). The peak height ratio range of heterozygous loci showing both alleles was 16% to 100%, with an average of 64%. However, when the 250 heterozygous loci that had a peak height ratio of 0% were included in the calculation, the average was reduced to 19% (Table 2.5).

2.4 Discussion

This study supports previous studies that showed increasing the number of PCR cycles will increase the sensitivity of detection for STR profiling of LTDNA samples. However, while 100pg has been noted as the upper limit for what may be considered a low template sample [14, 31], this research shows that when using the current generation multiplex kits, there may be less benefit to increasing the number of PCR cycles when this amount of DNA template is available for amplification. When 100pg of template was amplified using the PowerPlex® ESI 16 kit with an increased cycle PCR additional alleles were seen in the profiles. This is not surprising due to the increased sensitivity of the PowerPlex® ESI 16 kit, which has been shown to produce full profiles down to 62.5pg using its standard cycling protocol [12]. Furthermore, the standard protocol for this kit already utilizes 30 PCR cycles, as opposed to other commercially produced multiplex kits that use 28 or 29 cycles as the standard cycle number.

For the 100pg samples amplified using the standard cycling protocol, there were only 6 instances of allele drop out in the resulting 15 profiles, no locus drop out and only 2 spurious alleles seen overall. Of the two additional alleles, one was in the −4 position and one was in the +4 position to true alleles. When measured against their respective true alleles, the allele in the −4 position had a peak height ratio of 26%, while the allele in the +4 position had a peak height ratio of 36%. The position of the alleles could indicate that they are increased stutter rather than true drop in alleles. However, given the height of the additional peaks, particularly the +4 allele, it may be that they are true drop in. Furthermore, the additional alleles occurred at heterozygous loci, and in both cases the two correct alleles were also present. The presence of the ‘drop in’ alleles would normally indicate a potential mixture sample, with the additional alleles not part of the major profile. However, the other 14 loci showed no additional minor alleles, which would indicate the likely drop in or artefact nature of these allele peaks. In comparison, the 100pg samples amplified with 34 cycles did not display any drop out. However, 32 additional peaks were seen across the 15 profiles, the majority of which were not in the stutter positions. Of the additional alleles, 10 were seen at homozygous loci, which could mean that the loci could be falsely interpreted as heterozygote loci. However, given that the peak height ratios of nine of the additional alleles were less

than 3% of the true allele, this is unlikely. One drop in allele had a peak height ratio of 10% compared to the true allele, but this additional allele was seen in the +4 stutter position, and would also be interpreted with caution. Overall, these results suggest that performing one standard cycle PCR is preferable to performing one increased cycle reaction when 100pg of template are available for amplification because of the large reduction in allele drop in.

When the template amount is reduced, this research demonstrates that there is significant benefit to increasing the number of PCR cycles for STR typing in terms of the increased amount of information seen in the resulting profiles. With a starting template amount of 25pg, increasing the number of PCR cycles resulted in a 22% increase in the number of correct loci seen overall compared to the standard cycle profiles. Both allele and locus drop out were markedly reduced and the peak heights and peak height ratios both improved. However, as with the 100pg samples, increasing the number of PCR cycles for the amplification of 25pg did result in more drop in alleles seen in the profiles. Compared to the standard cycle profiles, which did not show any additional alleles, the increased cycle profiles displayed six loci with additional alleles. Of these additional alleles, four were seen at heterozygous loci, with three of these loci also containing both true alleles. The fourth locus did display allele drop out in conjunction with the allele drop in, so it is possible this locus would be interpreted incorrectly. The peak height ratio of the drop in allele compared to the remaining allele was only 9%, indicating that this would be interpreted with caution. However, if the drop in allele was considered not part of the major profile, the locus could then be falsely interpreted as homozygous. Of the two additional alleles that occurred at homozygous loci, one occurred in the −4 stutter position but was not removed by the stutter filter as it had a peak height ratio of 29%. The other was in the +4 stutter position, with a peak height ratio of 7%. If an increased cycle procedure were implemented for LTDNA amounts as low as 25pg, the profile interpretation would need to accommodate the chance of an allele resulting from allele drop in.

For both starting template amounts, the data show that splitting the sample into three aliquots and constructing a consensus profile did not result in the most informative profile compared with a profile where the DNA extract was amplified in one reaction. While the consensus profile approach did eliminate allele drop in, all other measures of profile quality were improved when the sample was not split. The original purpose of the Biological Model approach was to eliminate spurious alleles from the final consensus profile and give confidence that the final profile contains only the alleles of the actual contributor. The former was demonstrated by our results, with no additional alleles in the consensus profiles. This is important, as additional alleles in the profile can result in an incorrect interpretation, where either a homozygous locus is interpreted as a heterozygous locus or, if the drop in occurs in conjunction with a drop out, the wrong genotype may be assigned for that locus. This could have serious ramifications for casework, as errors in the profile could then lead to false inclusion or exclusion of suspects, or false matches if the profile is subjected to a database search.

The consensus profile results showed that a large amount of information was lost when the starting template was divided for amplification. This was especially evident in the 25pg samples. Compared to the profiles obtained with the full 25pg using a 34-cycle reaction, the consensus profiles showed a notable increase in allele and locus drop out. When the entire sample was amplified with 34 PCR cycles, 67% of the loci were complete and correct, 25% were heterozygous loci that showed only one allele, 5% showed complete locus drop out and 3% showed allele drop in. When the 25pg was split for amplification, the resulting consensus profile showed only 29% of the loci as complete and correct, 38% were heterozygous loci that showed only one allele and 33% displayed complete locus drop out. This loss of information that occurs when the sample is divided for amplification is not surprising, since the starting template amount in each of the split samples is barely more than the DNA that is available from a single cell, and that is only if the starting 25pg is divided equally into thirds. In reality, any of the three aliquots could contain less than a single copy of the genome. Furthermore, if the majority of the 25pg template happens to end up in one aliquot, then the consensus profiling method may effectively eliminate much information that would be gained from that aliquot's profile because it does not appear in one of the other aliquot profiles.

While repeatability is an important measure of reliability, the fact that so much information is lost in the attempt to repeat the results would suggest that the consensus profiling method may not be giving the most informative profile for samples with such a low level of starting template. It could then be argued that the gain of having confidence that no allele drop in has occurred is not sufficient compensation for the loss of profile information. This is particularly so given the increased occurrence of stutter in the split profiles, since a stutter and drop in are in fact both incorrect alleles. The results from the 25pg split samples show 10 additional alleles in the −4 stutter position, 1 in the +4 stutter position and 4 in other positions. As a consequence, many of the incorrect alleles being eliminated from the consensus profile are likely increased stutter, which is not actually seen in such high amounts in the non-split profiles (six additional alleles overall, two of which are in the −4 stutter position, one in the +4 stutter position, and three in random positions). Based on the allele drop in results, the use of a higher stutter filter for the −4 position and implementation of a +4 stutter filter would significantly reduce the incidence of apparent allele drop in. Therefore, a profile interpretation method that accommodates the increased stutter may be warranted.

It should be noted that, since allele and locus drop out and allele drop in still occurred when a low template DNA sample was amplified in a single reaction, a robust statistical analysis model that takes the stochastic effects into consideration must be applied to the data. A statistical analysis taking these stochastic effects into consideration should, of course, also be applied to the consensus profiles. It is noteworthy that this study was confined to single source samples. Interpretation of mixture profiles generated from LTDNA samples deriving from more than one individual would be more complex. Sample degradation or the presence of PCR inhibitors – issues commonly seen with low template samples – would further complicate profile interpretation. However, this study has shown that a consensus profile from a split single source sample contains considerably less information than a single profile from a non-split sample. It would, therefore, be preferable to build a statistical model that can be applied to the single LTDNA profile since this should provide the most information.

2.5 Conclusion

Overall, this study has demonstrated that performing standard cycling STR typing on non-split DNA extracts will result in profiles with a higher percentage of total loci compared with the consensus profiling technique. Increasing the number of PCR cycles improves the sensitivity of the reaction compared with a standard cycle PCR. However, samples containing template amounts on the upper limits of what would be considered low template DNA may not actually benefit from the increased amplification because of the additional alleles, either drop in or stutter, that can appear in the profile. The repeat nature of the consensus profiling method does eliminate the problem of allele drop in seen with an increased number of PCR cycles, which has important implications for casework. However, consensus profiling also results in the least informative profiles due to increased allele or locus drop out. It also results in more 'incorrect' alleles in the individual profiles used to obtain the consensus profile as a result of increased stutter. Simply performing a single standard cycle PCR on the entire sample produced the most complete profiles when 100pg of starting template are available for amplification. When only 25pg of template are available, it would be beneficial to amplify the entire extract with an increased cycle PCR in terms of acquiring a profile with the most information possible. While this must be balanced against the possibility of drop in, it is important to realize that increased stutter alleles that are likely to appear in the split sample profiles are also incorrect alleles and are more likely to be reproducible than 'random' allele drop ins.

Performing a single STR reaction from the whole low template sample does eliminate any chance of repeating the profile. In this sense, consensus profiling may be preferred because the results are seen as repeatable. However, the impression of repeatability gained by the consensus profiling method must be balanced against the notable loss of information that occurs when a LTDNA sample is divided for amplification. While consensus profiling does have its benefits, the method may not be producing the most informative STR profiles for samples where the template amount is limited.

CHAPTER 3

LINEAR AMPLIFICATION OF TARGET PRIOR TO PCR FOR IMPROVED LOW TEMPLATE DNA RESULTS

ADDENDUM

Work from this chapter has been accepted for publication in *BioTechniques*.

Portions of this work were presented at the 25th World Congress of the International Society for Forensic Genetics 2013.

3.1 Introduction

DNA profiling has become a powerful investigative tool and a compelling form of evidence when presented in court. Current profiling techniques which utilise the PCR and CE to type STR loci are extremely sensitive, allowing for as little as 200pg of DNA to be routinely analysed with various commercial kits [7-11]. However due to the sensitivity of the technique there has come an increased attempt to profile even smaller amounts of DNA.

One of the most popular methods for increasing the sensitivity of detection is the LCN technique, which employs an increased number of PCR cycles [14]. Other methods for increasing sensitivity can include post-PCR purification [5, 44] or modifying the CE injection conditions [3, 5]. These methods have been shown to increase the number of alleles seen in a profile compared with profiles obtained using conventional methods. However, exaggerated stochastic effects such as peak height imbalance, allele and locus drop out, increased stutter and allele drop in are commonly observed and can cause interpretation difficulties [2-6, 44].

Methods, such as WGA and nested PCR, have been suggested as potential ways to improve the yield of low level samples prior to STR analysis. Various WGA techniques have demonstrated improved allele detection but in most cases large stochastic effects were still observed [114, 115, 119-121, 126]. Nested PCR also has shown improved STR profile results [126, 168]. However, nested PCR still relies on an exponential amplification and thus preferential amplification of one allele in the initial PCR may amplify the stochastic effects observed after the second reaction, particularly increased stutter, allele drop out and heterozygote peak imbalance.

In this study a method was investigated to increase the DNA starting template amount available for STR analysis through a non-exponential first round PCR amplification. In this method, low template DNA samples were divided into two aliquots and a first round PCR is performed with one primer only, with the forward and reverse STR

primers placed in separate aliquots. The two aliquots were then pooled for a typical PCR with the forward and reverse primer pair. As a proof of concept, initial reactions targeted a single locus to determine if the Pre-PCR procedures could increase the amount of amplifiable target for the PCR without the additional complication of multiplexing. Experiments were then performed targeting multiple loci in a single reaction using primers targeting all loci in a commercial DNA profiling kit.

3.2 Methods

3.2.1 Sample preparation

This project was approved by the Bond University Human Research Ethics Committee (BUHREC), approval number RO743. Whole blood was provided by four anonymous donors with informed consent. DNA was extracted using the BioRobot EZ1® Workstation with the EZ1® DNA Blood Kits (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. DNA extracts were quantitated using the method outlined in section 2.2.1. Extracts were diluted to low template levels of 100pg/µl, 50pg/µl, 25pg/µl, 12.5pg/µl and 6.25pg/µl.

3.2.2 Single locus experiments

3.2.2.1 First round non-exponential PCR (Pre-PCR)

Initial PCRs were performed using HotStar Taq Mastermix Kits (QIAGEN) according to the manufacturer's instructions with the exception that reaction volumes were reduced to 10µl. Eleven reactions were performed for each starting template amount using the DNA from a single donor. For each template amount to be analysed, half of the total template was amplified with the forward primer for the vWA locus while half was amplified using the reverse primer. Sequences for the forward and reverse primers were obtained from published data [10] (Forward Primer = 5'-GCCCTAGTGGATGATAAGAATAATCAGTATGTG-3'. Reverse Primer = 5'-GGACAGATGATAAATACATA GGATGGATGG-3'). First round PCRs were performed for either 10 or 20 cycles. Amplification was performed using a GeneAmp®

PCR System 9700 (Life Technologies, Carlsbad, CA, USA). PCR cycling conditions were as follows: 95ºC for 15 minutes, then 10 or 20 cycles of 94ºC for 30 seconds, 55ºC for 30 seconds, 72ºC for 30 seconds, followed by a 72ºC hold for 15 minutes. Samples were then cooled and held at 4ºC. Forward and reverse primer reactions were pooled prior to the second vWA amplification.

3.2.2.2 Second round exponential PCR and capillary electrophoresis

Single-plex STR analysis was performed on the pooled samples using the HotStar Taq Mastermix Kits according to manufacturer's instructions. For each pooled sample 10µl was amplified with a 30-cycle PCR with the remaining 10µl amplified using a 35-cycle PCR. Amplification was performed using a GeneAmp® PCR System 9700. PCR cycling conditions were as follows: 95ºC for 15 minutes, then either 30 or 35 cycles of 94ºC for 30 seconds, 55ºC for 30 seconds, 72ºC for 30 seconds, followed by a 72ºC hold for 15 minutes as per manufacturer's instruction. Samples were then cooled and held at 4ºC. STR analysis also was performed on 100pg, 50pg, 25pg, 12.5pg and 6.25pg control samples that did not undergo the Pre-PCR processing using the same 30- or 35-cycle PCR conditions as described above.

CE was performed using a DNA High Resolution gel cartridge on a QIAxcel System (QIAGEN). The OM500 method (5kV for 500 seconds) was used for fragment separation. The QIAxcel system produces a digital gel image for each sample as well as providing fragment length and signal intensity information.

The DNA donor was known to be heterozygous for the vWA locus. Previous STR analysis using the PowerPlex® ESI 16 kit (Promega Corp, Madison, WI, USA) showed the sample was 13,16. Based on the placement of the primers used this should result in QIAxcel fragments of approximately 135bp and 147bp in length, respectively. Results were analysed to assess allele drop out (ADO) and locus drop out (LDO) levels using a 50 RFU detection threshold. The mean peak height and mean peak height ratio (PHR)

were calculated for each set of profiles obtained using the various Pre-PCR and second round PCR methods. The peak height ratios were calculated by dividing the height of the smaller peak by the height of the larger peak in the heterozygote pair. If allele drop out occurred at the locus, a peak height ratio of 0% was recorded. The peak height averages were calculated in two ways. First, the average of all the PHRs from sample profiles that showed both alleles was calculated. The second calculation included the PHRs from the profiles that showed both alleles, as well as the 0% PHRs recorded in profiles that showed allele drop out. Since the PHRs are used to measure how well both alleles at a locus amplified, including the 0% PHRs would help to indicate the efficiency of the entire reaction. If complete LDO occurred then locus this was not used in calculating the PHR average.

3.2.3 Multiplex experiments

3.2.3.1 First round non-exponential PCR (Pre-PCR)

Pre-PCRs were performed using PowerPlex® ESI 16 kit. Profiles from three individuals were obtained for each starting template amount. Reactions were performed according to the manufacturer's instructions with the following exceptions: reaction volumes were halved to 12.5µl and a 1µM primer mix containing unlabeled forward or reverse primers for the loci targeted in the ESI 16 kit was used instead of the provided primer mix. For each template amount to be analysed, half of the total template was amplified with the forward primer mix while half was amplified using the reverse primer mix. Forward and reverse primers were provided by Promega Corp. First round PCRs were performed for either 10 or 20 cycles. Amplification was performed using a GeneAmp® PCR System 9700 (Life Technologies, Carlsbad, CA, USA). PCR cycling conditions were as follows: 96ºC for 2 minutes, then 10 or 20 cycles of 94ºC for 30 seconds, 59ºC for 2 minutes, 72ºC for 90 seconds, followed by a 60ºC hold for 45 minutes. Samples were then cooled and held at 4ºC. Forward and reverse primer reactions were pooled prior to the second amplification.

3.2.3.2 Second round exponential PCR and capillary electrophoresis

Multiplex STR analysis was performed on the pooled samples using the PowerPlex® ESI 16 kit according to manufacturer's instructions. For each pooled sample 12.5µl was amplified with a 30-cycle PCR. Amplification was performed using a GeneAmp® PCR System 9700. PCR cycling conditions were as follows: : 96ºC for 2 minutes, then 30 cycles of 94ºC for 30 seconds, 59ºC for 2 minutes, 72ºC for 90 seconds, followed by a 60ºC hold for 45 minutes as per manufacturer's instruction. Samples were then cooled and held at 4ºC. STR analysis also was performed on 100pg, 50pg, 25pg, 12.5pg and 6.25pg control samples that did not undergo the Pre-PCR processing using the same 30-cycle PCR conditions as described above.

Electropherograms for all samples were obtained using the 3130 Genetic Analyser (Life Technologies). For each sample, a loading cocktail of 10µl Hi-Di^{TM} Formamide (Life Technologies) and 1µl of CC5 Internal Lane Standard 500 (Promega Corp) was mixed with 1µl of amplified product and denatured for three minutes at 95°C. After cooling, samples were injected on the 3130 using a 3 kV, 5-second injection as is the recommended PowerPlex® ESI 16 protocol. Data were analysed using Genemapper ID® software version 3.2.1 (Life Technologies) and PowerPlex® ESI 16 panel and bin files. A detection threshold of 50 RFU was used for analysis of all sample profiles.

Results were analysed to assess allele drop out (ADO) and locus drop out (LDO) levels. The mean peak height and mean peak height ratio (PHR) were calculated for each set of profiles obtained using the various Pre-PCR and second round PCR methods. The peak height ratios were calculated by dividing the height of the smaller peak by the height of the larger peak in the heterozygote pair. If allele drop out occurred at the locus, a peak height ratio of 0% was recorded. The peak height averages were calculated in two ways. First, the average of all the PHRs from sample profiles that showed both alleles was calculated. The second calculation included the PHRs from the profiles that showed both alleles, as well as the 0% PHRs recorded in profiles that showed allele drop out. Since the PHRs are used to measure how well both alleles at a locus amplified,

including the 0% PHRs would help to indicate the efficiency of the entire reaction. If complete LDO occurred then this locus was not used in calculating the PHR average.

3.3 Results and Discussion

The aim of this study was to develop a method that could reduce the stochastic sampling issues generally associated low template DNA analysis. It was proposed that dividing samples into two aliquots for a single-strand Pre-PCR amplification with either the forward or reverse primer followed by pooling the single strand products would provide an increased number of target molecules for STR analysis, with less stochastic effects than by solely performing exponential amplification. This is premised on the fact that the single primer reactions will generate more template in a linear or non-exponential manner. Since only one copy of the template is produced with each cycle the product generated would likely not be prey to exaggerated stochastic sampling effects, as there is a higher probability of a single primer annealing to the template, compared to traditional PCR where there is a requirement for both primers to sit down on the template for balanced amplification.

For each template amount, samples were subjected to single locus experiments as well as multiplex reactions. Samples were subjected to 10-cycle or 20-cycle Pre-PCR amplifications with the forward or reverse primers (one primer for single locus experiments, primer mix of all forward or reverse primers for the multiplex reactions) followed by a 30- or 35-cycle amplification with the primer pair. The results of the analyses were compared with control samples for each template amount that only underwent the 30- or 35-cycle PCRs.

3.3.1 Single locus experiments

3.3.1.1 30-cycle PCR

Pooled Pre-PCR products and control samples were initially subjected to a 30-cycle PCR since this is the middle of the range recommended by the HotStar Taq Mastermix Kit manufacturers (25-35 cycles recommended). Furthermore, many of the commercial STR kits use a similar cycle number. Results for each sample amount are summarized in Tables 3.1 and 3.2.

All samples subjected to the 30-cycle PCR without Pre-PCR processing failed to amplify. When the 10 cycle Pre-PCR was introduced improvements were seen in the number of loci with both alleles present for the 100pg, 50pg and 25pg samples. Results were further improved when the 20 cycle Pre-PCR was used (Figure 3.1).

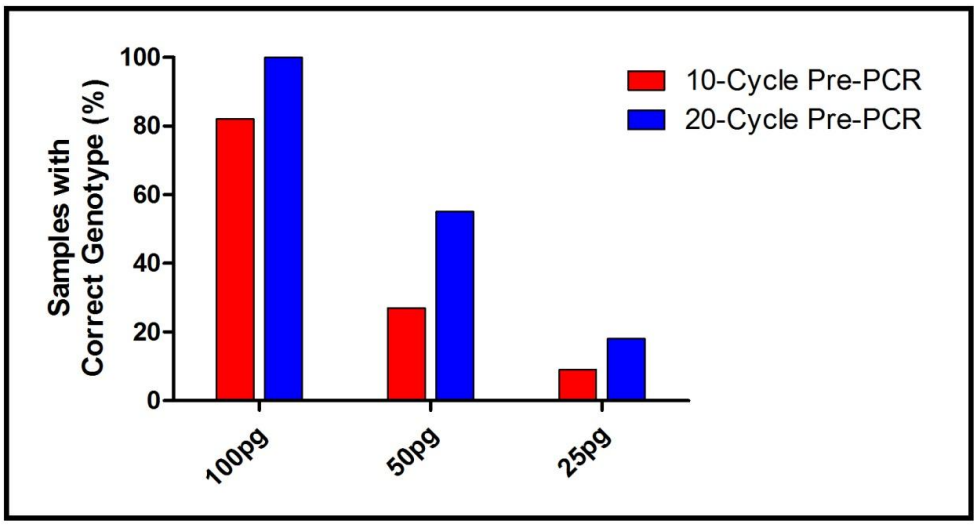

Figure 3.1 Correct genotypes recovered (30 cycle PCR). Results represent the percentage of samples recovered with the correct genotype after a 10- or 20-cycle Pre-PCR procedure followed by a 30-cycle PCR targeting a single locus. Eleven reactions were performed for each amplification method. Samples amplified without the Pre-PCR failed to detect any alleles.

With a 20-cycle Pre-PCR all 100pg sample profiles showed both alleles compared to 82% for the 10-cycle Pre-PCR samples. The average PHR for the 100pg samples amplified with a 20-cycle Pre-PCR was 75%, which is slightly lower than the 10-cycle Pre-PCR samples of 81%, likely due to the increased allele recovery of the 20-cycle Pre-PCR. The increase in allele recovery and the similar PHR averages in the Pre-PCR samples indicate that the linear amplification provided by the Pre-PCR was sufficient to increase the starting template amount without substantially introducing peak height imbalance.

The 50pg samples that underwent the 20-cycle Pre-PCR showed 55% of samples with both alleles, compared to 27% with the 10-cycle Pre-PCR, with a PHR average of 74%. However the remaining 45% of samples showed allele drop out, indicating that there is still some preferential amplification of particular alleles despite the linear amplification of the Pre-PCR. It is interesting to note that the PHR average for the 50pg samples amplified with the 10-cycle Pre-PCR is 90%, which is higher than the PHR average of 81% for the 100pg samples also amplified with a 10-cycle Pre-PCR. However, this difference is also likely due to the increased allele recovery in the 100pg samples. The majority of results for the 25pg and 12.5pg Pre-PCR samples showed partial profiles or complete LDO. This indicates that the Pre-PCR did not sufficiently increase the copy number of the target sequence for use as template for the 30-cycle PCR, so that both alleles could be observed using the QIAxcel detection system. It is possible that a greater number of Pre-PCR cycles would produce more complete allele, locus and profile results with these lower amounts of template.

Peak heights were generally higher in the 20-cycle samples compared to the 10-cycle samples. The exception was the 25pg samples, which showed a greater average peak height in the 10-cycle samples compared to the 20-cycle samples. While this is an unexpected result, the total allele recovery was higher in 25pg samples amplified with the 20-cycle Pre-PCR compared to 10-cycle samples, indicating that the 20-cycle Pre-PCR was more efficient despite the slight reduction in peak heights.

Table 3.1 30-Cycle single locus PCR amplification – allele recovery and peak heights

		Profiles with both alleles	Profiles with Allele Drop Out	Profiles with Locus Drop Out	Mean peak height (RFU) (Std. Dev.)
100pg:	No Pre-PCR	0% (0/11)	0% (0/11)	100% (11/11)	[a]
	10-Cycle Pre-PCR	82% (7/11)	18% (4/11)	0% (0/11)	79 (22)
	20-Cycle Pre-PCR	100% (11/11)	0% (0/11)	0% (0/11)	216 (81)
50pg:	No Pre-PCR	0% (0/11)	0% (0/11)	100% (11/11)	[a]
	10-Cycle Pre-PCR	27% (3/11)	9% (1/11)	64% (7/11)	72 (22)
	20-Cycle Pre-PCR	55% (6/11)	45% (5/11)	0% (0/11)	111 (34)
25pg:	No Pre-PCR	0% (0/11))	0% (0/11)	100% (11/11)	[a]
	10-Cycle Pre-PCR	9% (1/11)	0% (0/11)	91% (10/11)	131 (34)
	20-Cycle Pre-PCR	18% (2/11)	55% (6/11)	27% (3/11)	74 (28)
12.5pg:	No Pre-PCR	0% (0/11)	0% (0/11)	100% (11/11)	[a]
	10-Cycle Pre-PCR	0% (0/11)	0% (0/11)	100% (11/11)	[a]
	20-Cycle Pre-PCR	0% (0/11)	18% (2/11)	82% (9/11)	66 (4)
6.25pg:	No Pre-PCR	0% (0/11)	0% (0/11)	100% (11/11)	[a]
	10-Cycle Pre-PCR	0% (0/11)	0% (0/11)	100% (11/11)	[a]
	20-Cycle Pre-PCR	0% (0/11)	0% (0/11)	100% (11/11)	[a]

[a] Mean peak height not available as zero alleles appeared across all samples

Table 3.2 30-Cycle single locus PCR amplification – peak height ratios

		ADO (0%) Not Included In The PHR Calculation			ADO (0%) Included In The PHR Calculation		
		n[a]	**Mean PHR**	**Std. Deviation**	**n[b]**	**Mean PHR**	**Std. Deviation**
100pg:	No Pre-PCR	0	-	-	0	-	-
	10-Cycle Pre-PCR	7	81%	13%	11	51%	42%
	20-Cycle Pre-PCR	11	75%	13%	11	75%	15%
50pg:	No Pre-PCR	0	-	-	0	-	-
	10-Cycle Pre-PCR	3	90%	9%	4	54%	50%
	20-Cycle Pre-PCR	6	74%	11%	11	41%	40%
25pg:	No Pre-PCR	0	-	-	0	-	-
	10-Cycle Pre-PCR	1	*	*	1	*	*
	20-Cycle Pre-PCR	2	83%	9%	8	21%	39%
12.5pg:	No Pre-PCR	0	-	-	0	-	-
	10-Cycle Pre-PCR	0	-	-	0	-	-
	20-Cycle Pre-PCR	0	-	-	2	0%	0%
6.25pg:	No Pre-PCR	0	-	-	0	-	-
	10-Cycle Pre-PCR	0	-	-	0	-	-
	20-Cycle Pre-PCR	0	-	-	0	-	-

n^a is the number of samples with both alleles present out of 11 total samples
n^b is the number of samples with at least one allele present out of 11 total samples
- PHR average and Std. deviation could not be calculated as zero alleles were present
*PHR average and Std. deviation could not be calculated as only one allele pair present

3.3.1.2 35-cycle PCR

The number of 30-cycle samples that showed complete LDO indicated that this combination of PCR chemistry and the QIAxcel system is not as sensitive as other fluorescence based STR kits and CE detection methods which have shown results with the examined template amounts amplified with 30 PCR cycles [12, 13]. As a consequence, samples were further analysed using a 35-cycle PCR. The results are summarized in Tables 3.3 and 3.4.

More alleles were recovered with the 35-cycle experiments. For all template amounts, implementing either of the Pre-PCR procedures followed by the 35-cycle amplification produced a higher percentage of samples with both alleles compared to the control samples. Furthermore, the 20-cycle Pre-PCR samples showed a higher percentage of profiles with both alleles compared to 10-cycle Pre-PCR samples (Figure 3.2) Example QIAxcel digital gel images for each template amount can be seen in Figures 3.3 to 3.7.

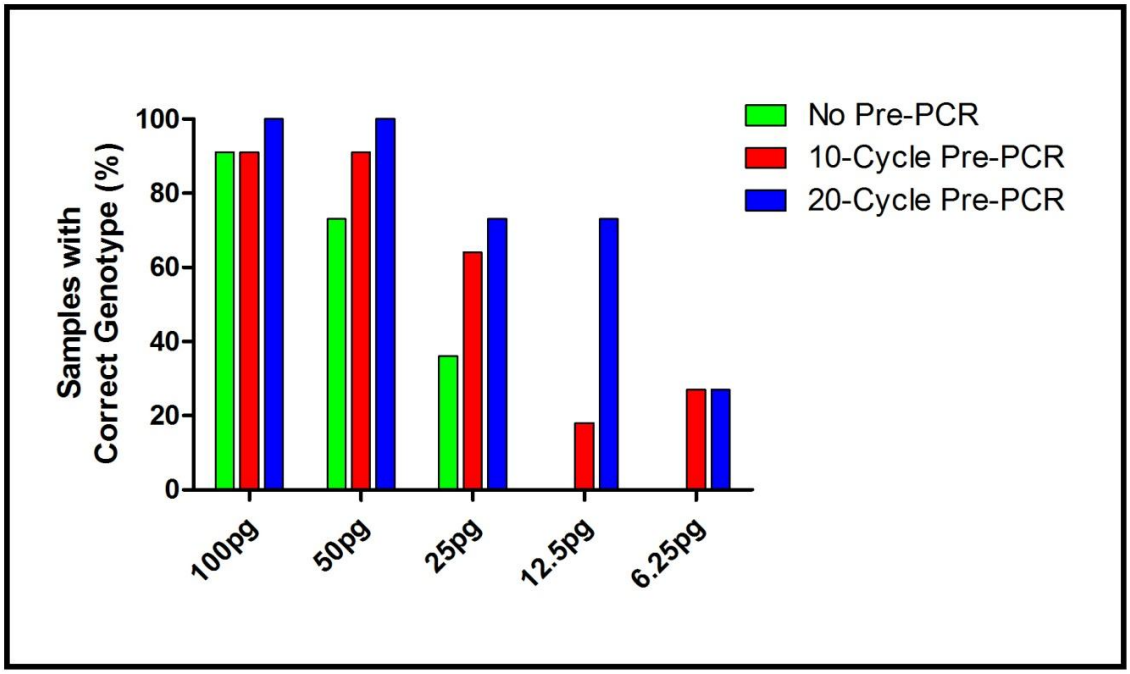

Figure 3.2 Correct genotypes recovered (35 cycle PCR). Results represent the percentage of samples recovered with the correct genotype after a single locus 35-cycle PCR with and without Pre-PCR treatment. Eleven reactions were performed for each amplification method.

With the 20-cycle Pre-PCR, all 100pg and 50pg samples showed both alleles. In comparison, 91% of 100pg and 50pg samples showed both alleles with 10-cycle Pre-PCR and 91% and 73% respectively displayed both alleles without Pre-PCR amplification.

The most notable differences were seen in the lower starting template samples. With 25pg starting template only 36% of control samples showed both alleles. When the Pre-PCR procedures were used the number of samples with both alleles increased to 64% with the 10-cycle Pre-PCR and 73% with the 20-cycle Pre-PCR. With 12.5pg starting template, none of the control samples showed both alleles. However, introducing the Pre-PCR techniques increased the recovery to 18% for 10 cycle samples and 73% for 20-cycle samples and an increased number of Pre-PCR cycles may improve this further. The 6.25pg samples that were subjected to the 20-cycle Pre-PCR showed only 27% of profiles with both alleles while 64% showed partial profiles. The 10-cycle Pre-PCR samples also showed 27% of profiles with both alleles but only 46% with partial profiles. Without the Pre-PCR, the 6.25pg samples did not produce any profiles with both alleles and only produced 18% with partial profiles.

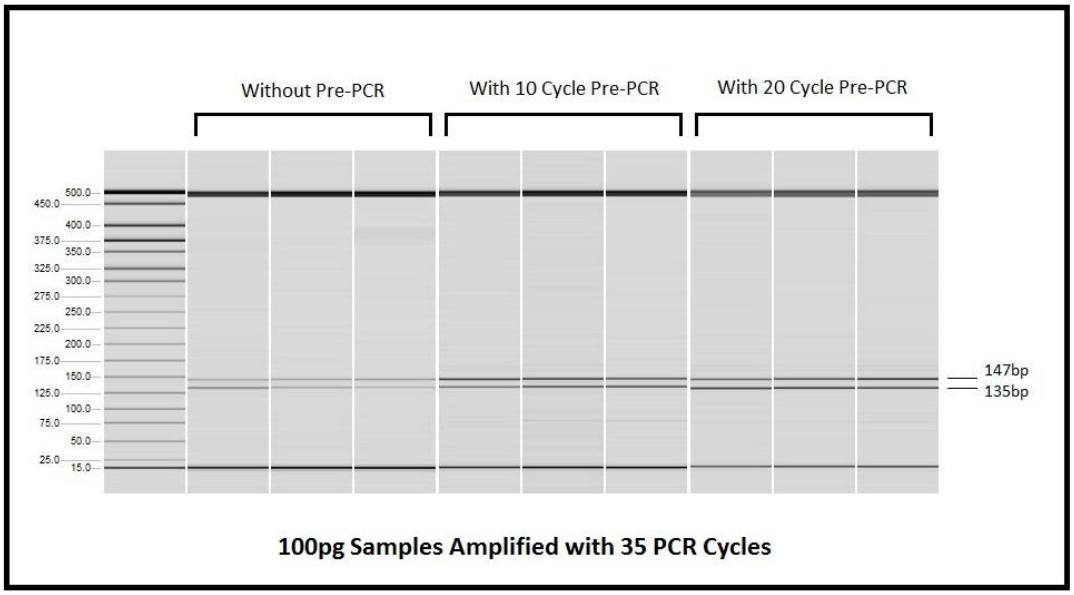

Figure 3.3 Example QIAxcel digital gel image from 100pg starting template amplified with 35 PCR cycles with and without Pre-PCR processing. Bands seen at 135bp and 147bp represent the 13 and 16 alleles of the WVA locus.

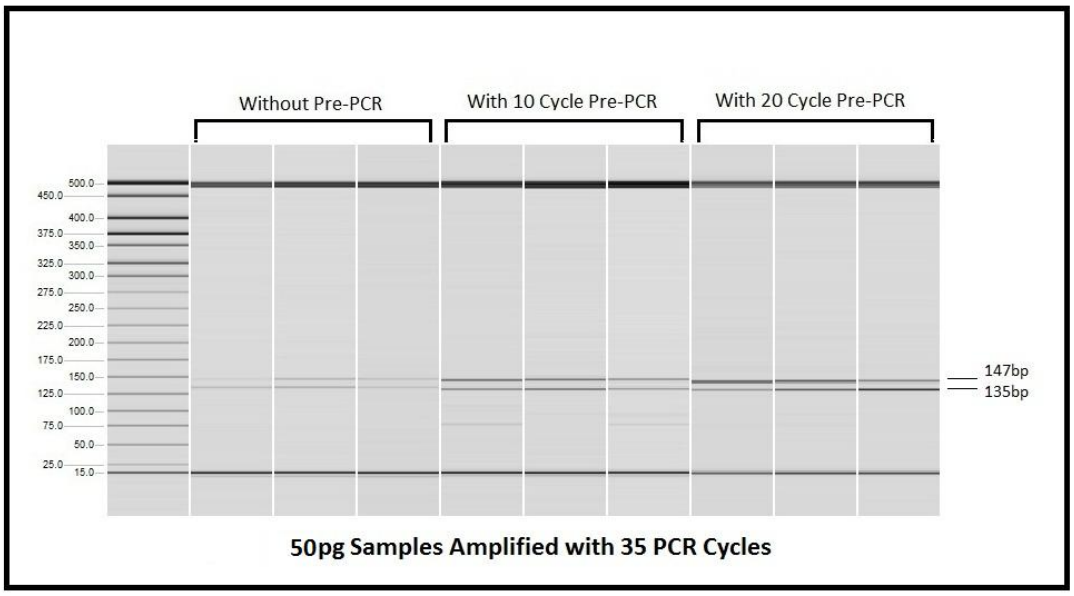

Figure 3.4 Example QIAxcel digital gel image from 50pg starting template amplified with 35 PCR cycles with and without Pre-PCR processing. Bands seen at 135bp and 147bp represent the 13 and 16 alleles of the WVA locus. Additional faint bands of less than 100bp can be seen in the first and third samples amplified with the 10 cycle Pre-PCR likely due to primer dimer.

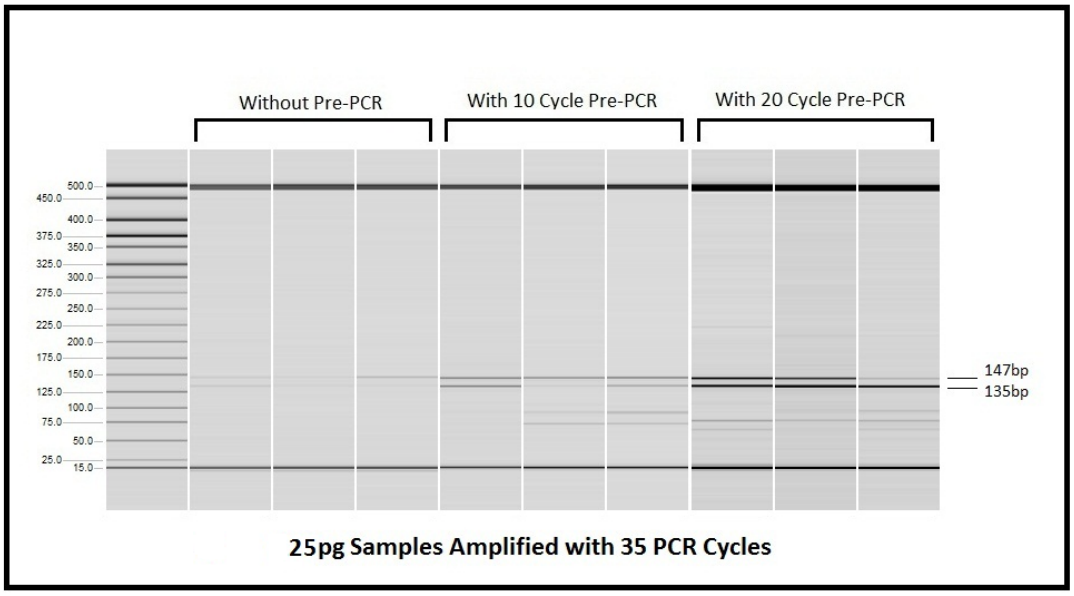

Figure 3.5 Example QIAxcel digital gel image from 25pg starting template amplified with 35 PCR cycles with and without Pre-PCR processing. Bands seen at 135bp and 147bp represent the 13 and 16 alleles of the WVA locus. Additional faint bands of less than 100bp can be seen in the samples amplified with both Pre-PCR procedures likely due to primer dimer.

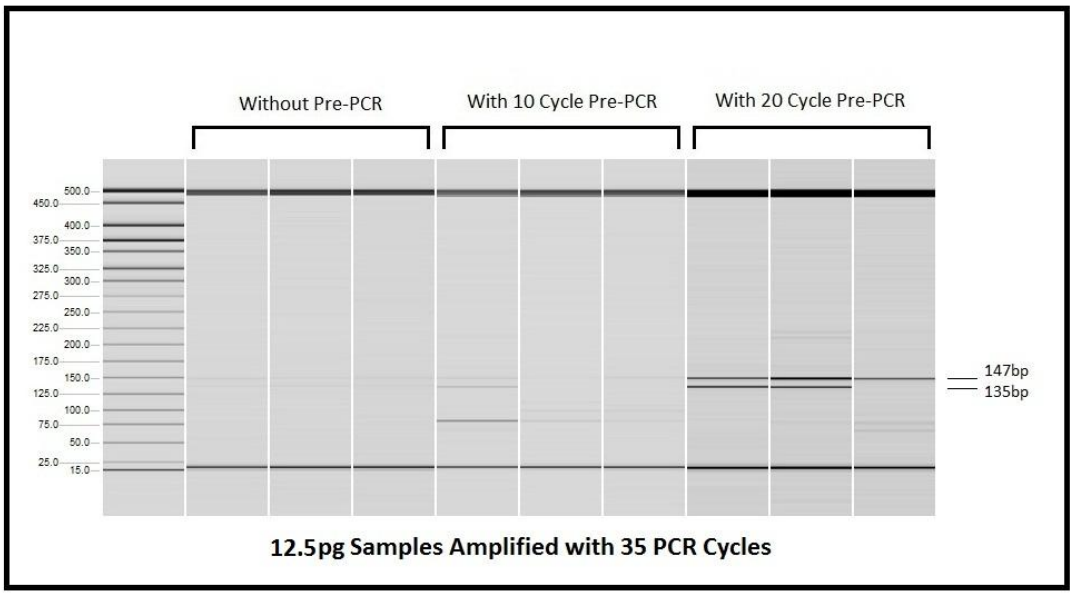

Figure 3.6 Example QIAxcel digital gel image from 12.5pg starting template amplified with 35 PCR cycles with and without Pre-PCR processing. Bands seen at 135bp and 147bp represent the 13 and 16 alleles of the WVA locus. Additional faint bands of less than 100bp can be seen in the samples amplified with both Pre-PCR procedures likely due to primer dimer.

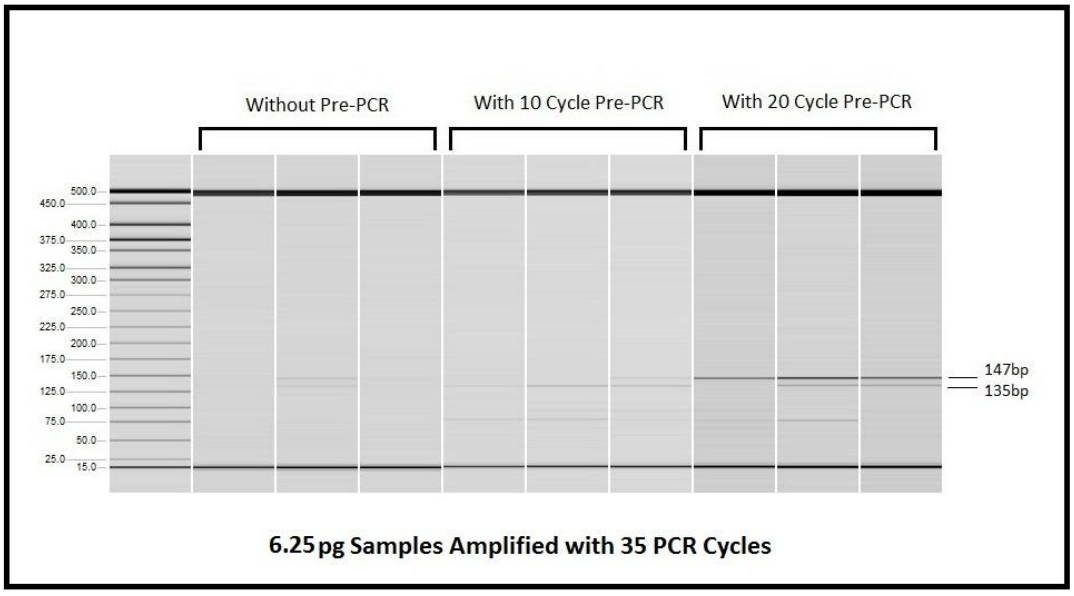

Figure 3.7 Example QIAxcel digital gel image from 6.25pg starting template amplified with 35 PCR cycles with and without Pre-PCR processing. Bands seen at 135bp and 147bp represent the 13 and 16 alleles of the WVA locus. Additional faint bands of less than 100bp can be seen in the samples amplified with both Pre-PCR procedures likely due to primer dimer.

Table 3.3 35-Cycle single locus PCR amplification – allele recovery and peak heights

		Profiles with both alleles	Profiles with Allele Drop Out	Profiles with Locus Drop Out	Mean peak height (RFU) (Std. Dev.)
100pg:	No Pre-PCR	91% (10/11)	9% (1/11)	0% (0/11)	331 (165)
	10-Cycle Pre-PCR	91% (10/11)	9% (1/11)	0% (0/11)	1071 (407)
	20-Cycle Pre-PCR	100% (11/11)	0% (0/11)	0% (0/11)	1479 (466)
50pg:	No Pre-PCR	73% (8/11)	27% (3/11)	0% (0/11)	199 (81)
	10-Cycle Pre-PCR	91% (10/11)	9% (1/11)	0% (0/11)	642 (413)
	20-Cycle Pre-PCR	100% (11/11)	0% (0/11)	0% (0/11)	1059 (485)
25pg:	No Pre-PCR	36% (4/11)	27% (3/11)	36% (4/11)	165 (44)
	10-Cycle Pre-PCR	64% (7/11)	27% (3/11)	9% (1/11)	470 (225)
	20-Cycle Pre-PCR	73% (8/11)	27% (3/11)	0% (0/11)	825 (404)
12.5pg:	No Pre-PCR	0% (0/11)	18% (2/11)	82% (9/11)	110 (25)
	10-Cycle Pre-PCR	18% (2/11)	36% (4/11)	46% (5/11)	274 (130)
	20-Cycle Pre-PCR	73% (8/11)	27% (3/11)	0% (0/11)	527 (337)
6.25pg:	No Pre-PCR	0% (0/11)	18% (2/11)	82% (9/11)	85 (50)
	10-Cycle Pre-PCR	27% (3/11)	46% (5/11)	27% (3/11)	246 (120)
	20-Cycle Pre-PCR	27% (3/11)	64% (7/11)	9% (1/11)	363 (215)

Table 3.4 35-Cycle single locus PCR amplification – peak height ratios

		ADO (0%) Not Included In The PHR Calculation			ADO (0%) Included In The PHR Calculation		
		n[a]	**Mean PHR**	**Std. Deviation**	**n[b]**	**Mean PHR**	**Std. Deviation**
100pg:	No Pre-PCR	10	79%	19%	11	72%	30%
	10-Cycle Pre-PCR	10	83%	14%	11	75%	28%
	20-Cycle Pre-PCR	11	79%	6%	11	79%	6%
50pg:	No Pre-PCR	8	70%	19%	11	51%	37%
	10-Cycle Pre-PCR	10	70%	28%	11	63%	34%
	20-Cycle Pre-PCR	11	68%	20%	11	68%	20%
25pg:	No Pre-PCR	4	91%	7%	7	52%	49%
	10-Cycle Pre-PCR	7	62%	18%	10	44%	34%
	20-Cycle Pre-PCR	8	65%	28%	11	47%	38%
12.5pg:	No Pre-PCR	0	-	-	2	0%	0%
	10-Cycle Pre-PCR	2	38%	16%	6	13%	21%
	20-Cycle Pre-PCR	8	55%	22%	11	40%	32%
6.25pg:	No Pre-PCR	0	-	-	2	0%	0%
	10-Cycle Pre-PCR	3	58%	22%	8	22%	32%
	20-Cycle Pre-PCR	3	32%	4%	10	10%	16%

n^a is the number of samples with both alleles present out of 11 total samples
n^b is the number of samples with at least one allele present out of 11 total samples
- PHR average and Std. deviation could not be calculated as zero alleles were present

Across all template amounts, the average peak height was greater with the Pre-PCR procedures compared to control samples. Furthermore, for each template amount, the 20-cycle Pre-PCR produced higher peaks than seen in the 10-cycle Pre-PCR samples.

For the 100pg and 50pg starting templates, PHR averages for the control, 10-cycle and 20-cycle samples were all very similar despite the increase in allele recovery and peak height (79%, 83% and 79% respectively for the 100pg samples and 71%, 70% and 68% respectively for the 50pg samples when looking at profiles that showed both alleles, and 72%, 79% and 79% respectively for the 100pg samples and 51%, 63% and 68% respectively for 50pg samples when the 0% PHRs were included in the calculation). These results indicate that the Pre-PCR is providing more template copies for STR analysis without considerably introducing peak height imbalance.

For the 25pg samples there was a reduction in the PHR average for samples with both alleles present when the Pre-PCR methods were introduced, from 91% for control samples down to 62% for 10-cycle samples and 65% for 20-cycle samples. This reduction in PHR average is likely because the Pre-PCR step allows for additional alleles to be detected that would not normally be seen in the control samples. Indeed there was a was an increase of 28% in the number of samples that showed both alleles in the 10-cycle Pre-PCR samples compared to the control samples, and an increase of 37% for the 20-cycle Pre-PCR samples compared to the control samples. These additional small peaks seen in the Pre-PCR samples would have reduced the peak height ratios, whereas the same alleles would likely have dropped out completely in the control samples and therefore not contributed to the peak height ratio calculations.

Some additional bands were seen in the QIAxcel profiles. Of the 350 total samples 48 additional alleles were noted. All but 2 of these additional alleles were seen in the Pre-PCR treated samples. The two additional alleles were seen in the same profile. Of the 46 additional alleles seen in Pre-PCR samples, all except one were seen in the 35-cycle

samples. However, the fragment length range for the alleles of the vWA locus is 123bp to 171bp and 47 of the 48 additional bands were within the range of 61bp to 108bp. The remaining additional band was 358bp in length. Since most of these fragments are shorter than the smallest possible allele for the locus and almost all are only present in the Pre-PCR samples they are likely a product of primer dimer from the excess primers in the reaction rather than true contamination. As such they would not be likely to be detected as alleles or even artefacts in a well-developed and balanced fluorescent STR kit.

3.3.2 Multiplex experiments

Results for the multiplex reactions also showed improvements in the total number of alleles recovered with the implementation of the Pre-PCR procedures (Figure 3.8). Details of all results can be seen in Tables 3.5 and 3.6. For all template amounts, more loci showed both alleles within each multiplex profile with the Pre-PCR than control samples amplified with the ESI 16 kit alone. The 100pg and 12.5pg samples showed a greater number of loci with both alleles correct in the 20-cycle Pre-PCR compared to the 10-cycle Pre-PCR. The 50pg, 25pg and 6.25pg samples showed a slightly higher number of loci with both alleles correct with the 10-cycle Pre-PCR compared to the 20-cycle Pre-PCR. However, for the 25pg samples the number of alleles recovered in total was greater with the 20-cycle Pre-PCR.

Peak heights were also greater in the Pre-PCR samples compared to the controls. Despite the increase in allele recovery and peak height, the peak height ratios did not differ greatly for the control, 10-cycle and 20-cycle Pre-PCR samples with 100pg, 50pg and 25pg starting templates (71%, 59% and 64% respectively for the 100pg samples, 72%, 65% and 76% respectively for the 50pg samples and 66%, 60% and 61% respectively for 25pg samples when looking at loci that showed both alleles, and 67%, 59% and 64% respectively for the 100pg samples, 55%, 55% and 58% respectively for 50pg samples and 16%, 38% and 32% respectively for 25pg samples when the 0% PHRs were included in the calculation). This indicates that neither Pre-PCR procedure introduced further stochastic variation.

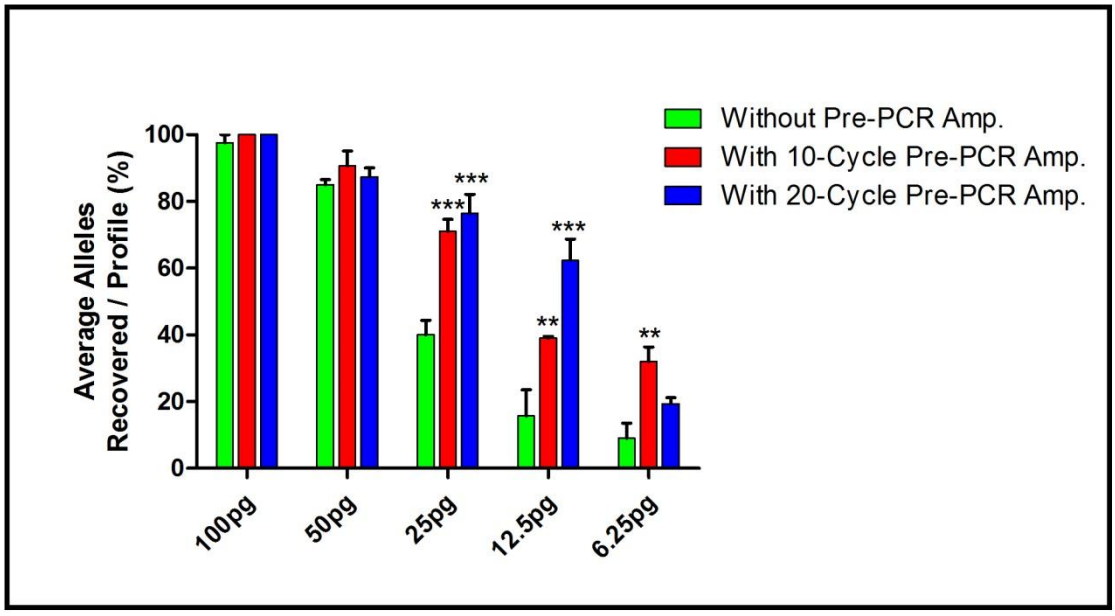

Figure 3.3 Correct alleles recovered in multiplex samples amplified with and without Pre-PCR processing. Results represent the average percentage of alleles recovered per 16-locus multiplex PCR sample. Three reactions were performed for each amplification method. Error bars represent standard error of the mean. A 2-way ANOVA with Bonferroni post-tests was performed to compare results from 10-cycle pre-PCR and 20-cycle pre-PCR samples to samples amplified without either pre-PCR procedure. As indicated,** represents a p-value of less than 0.01 and *** represents a p-value of less than 0.001.

The 12.5pg and 6.25pg control samples had higher peak height ratio averages compared to the 10- and 20- cycle Pre-PCR samples (93%, 41% and 66% respectively for the 12.5pg samples and 96%, 59% and 65% respectively for the 6.25pg samples when looking at loci that showed both alleles). However, like the single locus experiments, this is likely due to the increased allele recovery seen in the Pre-PCR samples. Furthermore, although the control samples have a higher mean PHR, the PHR averages (looking at loci with both alleles present) for the 12.5pg 20-Cycle Pre-PCR samples and the 6.25pg 10- and 20-cycle Pre-PCR samples are still at a reasonable level for single source samples.

With 100pg starting template the 10- and 20-cycle Pre-PCR produced STR profiles with all loci correct. However below this template amount, while there was an increase in allele recovery overall with the Pre-PCR, none of the control, 10- or 20-cycle Pre-PCR samples showed the complete multiplex STR profile. Since the second round PCR only used half of the pooled Pre-PCR sample results could be improved if the entire Pre-PCR sample was used. Further improvements may also be seen with an increase in the number of Pre-PCR cycles. The linear amplification of the Pre-PCR step has not introduced further stochastic variation compared to samples amplified without Pre-PCR processing when 20 Pre-PCR cycles were used. However, there is the possibility of introducing amplification bias if the number of Pre-PCR cycles is increased.

Table 3.5 Multiplex PCR amplification – allele recovery and peak heights

		Correct Loci	Loci with Allele Drop Out	Loci with Locus Drop Out	Mean peak height (RFU) (Std. Dev.)
100pg:	No Pre-PCR	96% (46/48)	4% (2/48)	0% (0/48)	204 (80)
	10-Cycle Pre-PCR	100% (48/48)	0% (0/48)	0% (0/48)	514 (500)
	20-Cycle Pre-PCR	100% (48/48)	0% (0/48)	0% (0/48)	742 (542)
50pg:	No Pre-PCR	77% (37/48)	19% (9/48)	4% (2/48)	118 (49)
	10-Cycle Pre-PCR	85% (41/48)	13% (6/48)	2% (1/48)	330 (302)
	20-Cycle Pre-PCR	79% (38/48)	19% (9/48)	2% (1/48)	372 (277)
25pg:	No Pre-PCR	23% (11/48)	38% (18/48)	39% (19/48)	94 (36)
	10-Cycle Pre-PCR	60% (29/48)	25% (12/48)	15% (7/48)	187 (132)
	20-Cycle Pre-PCR	58% (28/48)	36% (17/48)	6% (3/48)	197 (147)
12.5pg:	No Pre-PCR	10% (5/48)	15% (7/48)	75% (36/48)	78 (31)
	10-Cycle Pre-PCR	21% (10/48)	41% (20/48)	38% (18/48)	143 (100)
	20-Cycle Pre-PCR	48% (23/48)	36% (17/48)	17% (8/48)	156 (127)
6.25pg:	No Pre-PCR	4% (2/48)	13% (6/48)	83% (40/48)	70 (13)
	10-Cycle Pre-PCR	17% (8/48)	33% (16/48)	50% (24/48)	100 (52)
	20-Cycle Pre-PCR	13% (6/48)	17% (8/48)	70% (34/48)	140 (62)

Table 3.6 Multiplex PCR amplification – peak height ratios

		ADO (0%) Not Included In The PHR Calculation			ADO (0%) Included In The PHR Calculation		
		n[a]	**Mean PHR**	**Std. Deviation**	**n[b]**	**Mean PHR**	**Std. Deviation**
100pg:	No Pre-PCR	37	71%	19%	39	67%	23%
	10-Cycle Pre-PCR	39	59%	23%	39	59%	23%
	20-Cycle Pre-PCR	39	64%	24%	39	64%	24%
50pg:	No Pre-PCR	28	72%	18%	37	55%	35%
	10-Cycle Pre-PCR	32	65%	23%	38	55%	32%
	20-Cycle Pre-PCR	29	76%	20%	38	58%	37%
25pg:	No Pre-PCR	6	66%	10%	24	16%	29%
	10-Cycle Pre-PCR	21	60%	24%	33	38%	34%
	20-Cycle Pre-PCR	19	61%	23%	36	32%	35%
12.5pg:	No Pre-PCR	2	93%	6%	9	21%	41%
	10-Cycle Pre-PCR	4	41%	10%	24	7%	16%
	20-Cycle Pre-PCR	14	66%	23%	31	30%	37%
6.25pg:	No Pre-PCR	1	96%	0%	6	16%	39%
	10-Cycle Pre-PCR	4	59%	22%	20	12%	26%
	20-Cycle Pre-PCR	3	65%	10%	11	18%	31%

n^a is the number of loci with both alleles present out of 39 total heterozygous loci
n^b is the number of loci with at least one allele present out of 39 total heterozygous loci

3.4 Conclusion

Overall this research has demonstrated that improved STR profiles from samples with low levels of template can be obtained using a 10- or 20-cycle Pre-PCR amplification prior to a single locus or multiplex PCR with the 20-cycle Pre-PCR generally providing the highest percentage of profiles with both alleles. The 20-cycle Pre-PCR also produced profiles with higher average peak heights compared to 10-cycle Pre-PCR samples and control samples for almost all template amounts. The peak height ratios for the Pre-PCR amplified samples were not considerably different compared with control samples in the single locus experiments for the 100pg and 50pg template amounts amplified with 35 PCR cycles, and in the multiplex experiments using 100pg, 50pg and 25pg starting template, indicating that the linear amplification of the Pre-PCR was increasing the number of template copies available for the PCR without introducing substantial amplification bias for these template amounts. The PHRs were reduced for the single locus 25pg samples that underwent either Pre-PCR followed by a 35-cycle amplification and the 12.5pg and 6.25pg multiplex Pre-PCR samples compared to the control samples, likely due to the increased allele recovery in the Pre-PCR samples. The increased allele recovery seen in the 12.5pg and 6.25pg single locus Pre-PCR samples amplified with 35 cycles improved the PHRs compared to the controls since none of the samples showed both alleles in the control samples. However, for both the single locus and multiplex 12.5pg and 6.25pg Pre-PCR samples allelic imbalance was still present with considerable allele drop out and very low PHR averages when the allele drop-out loci are included in the calculation, indicating that the Pre-PCR, at least when limited to 20 cycles, is not sufficient to improve the number of template copies for the PCR amplification for such low level samples.

In these experiments, only half of the first-round Pre-PCR product was used as the template for the second-round PCR. While this was still shown to improve allele recovery, results may be further improved if the entire first round PCR product was used. Future work could therefore involve further reducing the volume of the first-round Pre-PCR or increasing the volume of the second round PCR so that all of the possible template could be used for the second PCR amplification. The use of fluorescence based

CE would also be useful for single locus PCR samples, as this would significantly increase the sensitivity and likely allow for more alleles to be seen in final profiles without the need for increasing the number of PCR cycles. Future research would also need to involve further development of the multiplex Pre-PCR amplification as multiplex STR profiling is a primary method of human identification used in forensic casework.

CHAPTER 4

WHOLE GENOME AMPLIFICATION

4.1 Introduction

Whole genome amplification has been proposed as a promising method for increasing the template copy number of limited quantity DNA samples prior to traditional DNA profiling [17]. Theoretically, WGA should be capable of copying all of the DNA in a representative fashion to produce large quantities of product for standard forensic analysis [17]. Several methods of WGA have been investigated including PCR based techniques [97-100] and Multiple Displacement Amplification (MDA) [116-118]. MDA, an isothermal amplification technique that utilizes φ29 DNA polymerase and random hexamer primers, has been shown to produce higher yields [109-113] and improved genome coverage [111, 113-115] compared to PCR based methods.

STR profiling on MDA product that used LTDNA as the starting template has been investigated [115, 124, 125]. In each case, exaggerated stochastic variation in the form of allele drop out was observed in the STR profiles when less than 1ng [115], 500pg [124] or 250pg [125] was used as the starting template. Despite this, results showed that the profiling success of LTDNA increases with MDA, with one study reporting an average of 7 more alleles being observed in WGA samples than non WGA samples from 10pg of starting template [115]. However, none of the LTDNA samples in that study, with or without WGA, produced STR profiles that had all alleles present [115]. Peak height imbalance, increased stutter and allele drop out are also commonly observed in many MDA samples from LTDNA starting templates [115, 124, 125].

Initial WGA experiments aimed to examine the ability of one commercial WGA kit – the GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Buckinghamshire, England) – to amplify low template DNA for forensic STR analysis. Modifications to the standard protocol also were examined to see if these changes could improve the MDA efficiency to result in a more complete forensic STR profile with reduced allelic imbalance compared to the manufacturer's protocol.

The first modification, called the “Cycling protocol” involved introducing heating and cooling cycles at the start of the MDA reaction. It was proposed that heating the reaction to 40°C would halt the reaction such that the amplification process could be re-initiated at another random site on the template when the sample was returned to 30°C. The second amendment, referred to as the “Split and Pool protocol”, involved dividing the MDA reaction into 4 aliquots prior to amplification, then pooling the aliquots prior to STR profiling. It was proposed that splitting the reaction then re-pooling for STR analysis could help balance any amplification bias that may be produced in each individual aliquot. The third variation involved denaturing only half of the DNA and was called the “Half Denatured protocol”. Previous work has shown that combining WGA product from two reactions, one where the DNA was denatured prior to the 30°C incubation and one where the DNA was not denatured, resulted in STR profiles with greater balance between alleles at heterozygous loci [129]. The Half Denatured protocol followed a similar method. However, denatured DNA was combined with DNA that was not denatured prior to the incubation so that the WGA reaction was performed in a single tube.

After the initial experiments described above were performed, a novel WGA kit called AT GenomiPhi (AT Kit) was provided by GE Healthcare for comparison to the GenomiPhi V2 DNA Amplification Kit. This kit is similar to the traditional GenomiPhi V2 DNA amplification kit in that it uses φ29 DNA polymerase to amplify genetic material in an isothermal reaction. However, in the AT kit novel hexamer primers containing 2-amino-deoxyadenosine and 2-thio-deoxythymidine are used to help reduce primer dimer formation and consequent non-specific amplification [169]. An additional polymerase cleaning step is also introduced to remove any contaminating DNA from the reagents [170].

A third WGA kit, the REPLI-g Mini Kit (QIAGEN, Hilden, Germany), was also assessed to determine which could best amplify LTDNA for forensic STR analysis. The AT kit was also used to amplify low template mixture samples with either equal input from both contributors (1:1 ratio) or major and minor contributions (9:1 ratio) to assess whether these WGA methods could improve allele recovery. Finally, the Cycling and

Split and Pool protocols were applied to the AT Kit to see if improvements could be made compared to the standard protocol.

4.2. Methods

4.2.1. Sample preparation

This project was approved by the Bond University Human Research Ethics Committee (BUHREC), approval number RO743. Whole blood samples were provided by four anonymous donors with informed consent. DNA was extracted using the BioRobot EZ1® Workstation with the EZ1® DNA Blood Kits (QIAGEN) according to the manufacturer's instructions. DNA extracts were quantified using the method outlined in section 2.2.1.

For the first group of experiments, extracts were diluted to 1ng/µl and 500pg/µl to be used as controls as well as low template levels of levels 60pg/µl, 30pg/µl, 12pg/µl and 6pg/µl. For the second group of experiments, 10ng/µl, 1ng/µl and 500pg/µl dilutions were generated as controls as well as low template samples of 100pg/µl, 50pg/µl, 25pg/µl and 10pg/µl. Two person mixture samples were also created. The first mixture, called "M1" contained 50pg/µl from both contributors such that the total concentration was 100pg/µl with a 1:1 ratio. The second mixture, "M2" contained 450pg/µl from one contributor and 50pg/µl from the second contributor, for a total concentration of 500pg/µl with a 9:1 ratio.

4.2.2 Whole genome amplification

In the first group of experiments ten of each 1ng/µl, 60pg/µl, 30pg/µl, 12pg/µl and 6pg/µl were amplified using the GenomiPhi V2 DNA Amplification Kit. The same dilutions were also amplified using the Cycling Protocol, the Split and Pool Protocol and the Half Denatured Protocol. Ten samples were amplified for each of the starting

template amounts with the various protocol amendments. Reactions were performed using a GeneAmp® PCR System 9700 (Life Technologies, Carlsbad, CA, USA).

In the Cycling Protocol, WGA reactions were set up according to the manufacturer's instructions then incubated with the following cycling conditions: 30 cycles of 30°C for 10 seconds then 40°C for 5 seconds, followed by a 30°C incubation for 2 hours. The reaction was deactivated with a 65°C incubation for 20 minutes then sampled were cooled and held at 4°C.

For the Split and Pool Protocol, samples were prepared as per the manufacturer's instructions, but were divided into 4 x 5µl aliquots for the 30°C incubation. The aliquots were then pooled after the amplification process.

In the Half Denatured protocol, 1µl containing entire template amount to be amplified was placed in a 0.2ml PCR tube with 9µl of GenomiPhi Sample Buffer. The sample was briefly mixed then 5µl was removed and placed in a separate 0.2ml tube on ice. The remaining 5µl was heat denatured at 95°C for 3 minutes then cooled to 4°C on ice. Aliquots containing the denatured DNA and the non-denatured DNA were combined and the reaction continued as per the manufacturer's protocol. All WGA samples from the second set of experiments were diluted 1:100 for STR analysis.

In the second set of experiments, five of each 10ng/µl, 100pg/µl, 50pg/µl, 25pg/µl and 10pg/µl dilutions were amplified using the REPLI-g Mini Kit and the GenomiPhi V2 DNA Amplification Kit according to the manufacturer's instructions. However, the recommended reaction volume was halved to 25µl for the REPLI-g kit. Amplification using the AT kit involved a three step process of Polymerase Cleaning, Template Preparation and Reaction Initiation. In the Polymerase Cleaning step, for each reaction to be performed, 7µl of water, 10µl of 2X Reaction Buffer, 0.8µl of AT oligo, and 0.4µl of Phi29 polymerase were combined in a 0.2ml PCR tube. Samples were incubated at 30°C for 1 hour then held at 4°C. In the Template Preparation step one volume of

required DNA template was combined with one volume of Denaturation Solution (0.4 M NaOH, 1 mM EDTA) and held on ice for 10 minutes. One volume of Neutralization Solution (0.4 M HCl, 0.6 M Tris, pH 7.5) was then added to halt the DNA denaturation process. In the Reaction Initiation step, 0.8µl of 10 mM dNTPs and 1µl of prepared DNA template were added to each cleaned Reaction Buffer sample and incubated at 30°C for 2.5 hours. All WGA reactions were performed using a GeneAmp® PCR System 9700. Five replicates of each mixture sample were also amplified using the AT Kit according to the manufacturer's instructions.

Two modified methods - the Cycling Protocol and the Split and Pool Protocol - were also applied to the AT Kit, with 10ng, 1ng, 100pg and 10pg input template amounts amplified in triplicate. All WGA samples from the second set of experiments were quantified in triplicate after the WGA reaction using SensiMix™ High Resolution Melt Kits. Samples were diluted to 500pg/µl as well as 1:100 for STR analysis.

4.2.3 Short tandem repeat analysis

STR analysis was performed on 1µl of each WGA dilution using the PowerPlex® ESI 16 Kits (Promega Corp, Madison, WI, USA) according to the manufacturer's instructions. Profiles were also obtained for 10 replicates of each 500pg/µl, 60pg/µl, 30pg/µl, 12pg/µl and 6pg/µl dilution as control samples that did not undergo WGA for the first set of experiments. Five replicates of each 500pg/µl, 100pg/µl, 50pg/µl, 25pg/µl and 10pg/µl dilution as well as both mixture samples were amplified as controls for the second set of experiments. Electropherograms for all samples were obtained using the 3130 Genetic Analyser (Life Technologies). For each sample a loading cocktail of 10µl Hi-Di™ Formamide (Life Technologies) and 1µl of CC5 Internal Lane Standard 500 (Promega Corp) was mixed with 1µl of amplified product and denatured for three minutes at 95°C. After cooling, samples were injected on the 3130 using a 3kv, 5 second injection as is the recommended PowerPlex® ESI 16 protocol. Data were analysed using Genemapper ID® software version 3.2.1 (Life Technologies) and PowerPlex® ESI 16 panels and bins files. A detection threshold of 100 RFU was used for analysis of all sample profiles.

All electropherograms were compared to a reference profile to determine if exaggerated stochastic sampling variation was present in the profiles. For the purpose of this study, exaggerated stochastic effects include preferential amplification of one allele of a heterozygous pair resulting in a peak height ratio of less than 60%, failure of one allele at a heterozygous locus to amplify resulting in allele drop out, complete amplification failure resulting in locus drop out and spurious contamination resulting in allele drop in.

For all profiles allele drop out (ADO), locus drop out (LDO), peak heights and peak height ratios (PHR) were recorded. PHRs were determined by dividing the height of the smaller peak in a heterozygote pair by the height of the larger peak. If allele drop out occurred at the locus, a peak height ratio of 0% was recorded. The peak height averages were calculated in two ways. First, the average of all the PHRs from sample profiles that showed both alleles was calculated. The second calculation included the PHRs from the profiles that showed both alleles, as well as the 0% PHRs recorded in profiles that showed allele drop out. Since the PHRs are used to measure how well both alleles at a locus amplified, including the 0% PHRs would help to indicate the efficiency of the entire reaction. If complete LDO occurred then this locus was not used in calculating the PHR average.

4.3 Results and Discussion

4.3.1GenomiPhi modifications

This study initially aimed to assess the ability of the GenomiPhi V2 DNA Amplification Kit to amplify low template DNA. Modifications to the standard protocol were also examined. The efficiency of the WGA reactions was assessed through multi-locus STR analysis. STR results for WGA samples were compared to a reference sample to determine if any allele or locus drop out had occurred. Peak heights and PHRs for each heterozygous locus were recorded. WGA sample profiles were also compared to profiles obtained using the same low level DNA starting template without WGA treatment to determine if allele recovery was improved with the prior amplification. Detailed results can be seen in Figure 4.1 and Tables 4.1 and 4.2.

STR analysis using 500pg genomic DNA starting template (the PowerPlex® ESI 16 recommended amount) without prior WGA produced, as expected, profiles with 100% allele recovery. Heterozygous loci showed well balanced peaks with a PHR average of 83% and an average peak height of 2157 RFU. All STR profiles from 1ng samples amplified with the standard and modified GenomiPhi methods showed all alleles recovered except for the standard protocol, which showed a single allele drop out in one profile. Each of the methods showed similar PHRs with averages for each method ranging from 68% to 76%, indicating that the standard WGA protocol and the modified methods were all amplifying the DNA in a representative fashion. STR profiles also showed alleles with similar peak heights. WGA samples were not quantified prior to STR analysis rather all WGA samples underwent a 1:100 dilution for STR analysis. Therefore, the similar peak heights indicate that, at least with 1ng starting template, all methods produced similar amounts of amplification product.

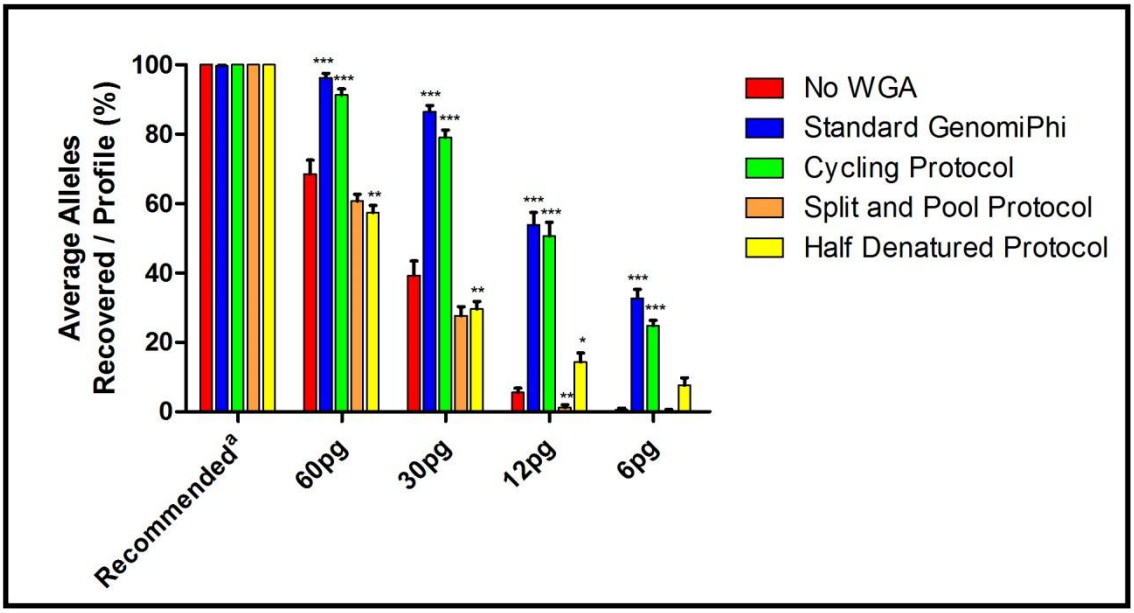

Figure 4.1 Correct alleles recovered in STR profiles of samples amplified with modified GenomiPhi protocols. Results represent the average percentage of alleles recovered from ten reactions for each WGA method. [a] For samples that underwent the various GenomiPhi protocols 1ng was used as the template for WGA. 1ng WGA results were compared to STR profiles that used the ESI 16 kit recommended template of 500pg. All WGA samples were diluted 1:100 for STR analysis. Error bars represent standard error of the mean. A 2-way ANOVA with Bonferroni post-tests was performed to compare the samples amplified using various WGA protocols to profiles obtained without prior WGA. As indicated, * represents a p-value of less than 0.05, ** represents a p-value of less than 0.01 and *** represents a p-value of less than 0.001.

Table 4.1 GenomiPhi with modified protocols – allele recovery

		% Mean Correct Alleles Per Profile[a] (Std. Dev.)	% Allele Drop Out[b]	% Locus Drop Out[c]
Rec[d]:	Without WGA	100% (0%)	0%	0%
	Standard	100% (1%)	1%	0%
	Cycling	100% (0%)	0%	0%
	Split and Pool	100% (0%)	0%	0%
	Half Denatured	100% (0%)	0%	0%
60pg:	Without WGA	68% (13%)	25%	19%
	Standard	96% (4%)	7%	1%
	Cycling	91% (5%)	15%	1%
	Split and Pool	61% (6%)	47%	16%
	Half Denatured	57% (7%)	50%	18%
30pg:	Without WGA	39% (13%)	33%	43%
	Standard	86% (6%)	26%	2%
	Cycling	79% (7%)	25%	8%
	Split and Pool	28% (6%)	38%	54%
	Half Denatured	30% (7%)	41%	49%
12pg:	Without WGA	5% (4%)	10%	90%
	Standard	54% (11%)	40%	27%
	Cycling	51% (13%)	45%	27%
	Split and Pool	1% (3%)	3%	97%
	Half Denatured	15% (8%)	24%	74%
6pg:	Without WGA	1% (1%)	1%	98%
	Standard	33% (8%)	43%	46%
	Cycling	25% (5%)	39%	54%
	Split and Pool	0% (1%)	1%	99%
	Half Denatured	8% (7%)	10%	87%

[a] Results represent the average of ten reactions per method.
[b] Results represent the percentage of loci with ADO of 150 heterozygous loci
[c] Results represent the percentage of loci with LDO of 160 total loci
[d] Rec = Recommended template amount, with 1ng used as the starting template for all WGA reactions and 500pg used as the starting template for STR analysis without WGA.

Table 4.2 GenomiPhi with modified protocols – peak heights and peak height ratios (PHR)

		Peak Heights			PHR - ADO (0%) Not Included			PHR - ADO (0%) Included		
		n[a]	Mean	Std. Deviation	n[b]	Mean	Std. Deviation	n[c]	Mean	Std. Deviation
Rec*[d]*:	Without WGA	310	2157	909	150	83%	12%	150	83%	12%
	Standard	309	4114	3446	149	73%	17%	150	73%	17%
	Cycling	310	3327	1932	150	72%	19%	150	72%	19%
	Split and Pool	310	4340	2019	150	76%	15%	150	76%	15%
	Half Denatured	310	3779	2697	150	68%	20%	150	68%	20%
60pg:	Without WGA	212	191	77	83	74%	14%	119	51%	36%
	Standard	298	3861	2878	139	45%	27%	149	42%	29%
	Cycling	284	3341	2830	125	44%	29%	148	37%	31%
	Split and Pool	188	1357	1804	54	37%	26%	124	16%	25%
	Half Denatured	178	2098	2558	47	39%	30%	122	15%	27%
30pg:	Without WGA	122	158	57	31	79%	18%	81	30%	40%
	Standard	265	3029	2939	108	38%	29%	147	29%	30%
	Cycling	246	3617	3101	99	40%	30%	138	29%	31%
	Split and Pool	86	919	1280	13	47%	25%	70	9%	21%
	Half Denatured	92	1211	1636	11	29%	23%	72	4%	13%
12pg:	Without WGA	17	137	40	1	87%	0%	16	5%	22%
	Standard	167	2269	2486	49	35%	27%	109	16%	25%
	Cycling	156	3808	3123	40	37%	26%	107	14%	24%
	Split and Pool	4	228	139	0	-	-	4	0%	0%
	Half Denatured	44	1521	2372	3	69%	25%	39	5%	19%
6pg:	Without WGA	2	122	19	0	-	-	1	0%	0%
	Standard	101	2833	2906	15	34%	30%	80	6%	18%
	Cycling	75	2747	3034	5	47%	30%	63	4%	15%
	Split and Pool	1	130	0	0	-	-	1	0%	0%
	Half Denatured	23	1212	2055	3	44%	13%	18	7%	18%

n[a] is the number of alleles recovered out of 310 total alleles per method

n[b] is the number of loci with both alleles present out of 150 total heterozygous loci

n[c] is the number of loci with at least one allele present out of 150 total heterozygous loci

[d] = Recommended template amount (1ng used as the starting template for all WGA reactions and 500pg used as the starting template for STR analysis without WGA).

Exaggerated stochastic variation was observed in all STR profiles from LTDNA without prior WGA. As expected, allele and locus drop out increased while peak heights decreased with reduced starting template amounts. When the various WGA methods were used to amplify the low template DNA samples, an overall increase in the number of alleles recovered per profile was observed. This was expected since previous studies have shown that WGA could improve allele recovery with LTDNA starting template [115, 125].

Results showed that the greatest allele recovery was achieved with the standard protocol followed by the Cycling protocol, with both protocols continually showing greater allele recovery compared to STR profiles from low template samples that did not undergo prior WGA (Figure 4.1). The Split and Pool and Half Denatured protocols consistently showed lower allele recovery compared to the standard and Cycling protocols, with the Split and Pool protocol generally showing the lowest efficiency of the methods. With 60pg and 30pg starting templates, the Half Denatured protocol showed lower allele recovery compared to samples that did not undergo WGA. However, the allele recovery was higher in the Half Denatured WGA samples with 12pg and 6pg starting template compared to samples without WGA. Across all low template amounts the Split and Pool protocol consistently showed less alleles recovered compared to samples that did not undergo prior WGA.

The low allele recovery for the various WGA protocols is likely due to the 1:100 dilution of WGA product prior to STR analysis. Since WGA reactions were not quantified prior to STR analysis the 1:100 dilutions may have reduced the template to sub optimal levels. It is therefore recommended that for further experiments the WGA samples be quantified prior to STR analysis so that sufficient DNA template can be added for optimal profiling results.

Overall the PHR averages for the LTDNA control samples without WGA were higher than the WGA samples when examining loci with both alleles present. This is likely because the various WGA protocols allow for additional alleles to be detected that

would not normally be seen in the control samples. Additional small peaks seen in the WGA samples may reduce the PHRs, whereas the same alleles would likely have dropped out completely in the control samples and therefore not contributed to the PHR calculations. Including the 0% ADO scores in the PHR calculation reduced the means, such that control and WGA samples showed similar results (Table 4.2).

PHRs were similar for all WGA methods across all LTDNA starting templates, with averages generally less than 50% for all protocols using both PHR calculation methods. The exception to this was the 12pg samples amplified with the Half Denatured protocol which had a PHR mean of 69% when ADO was not included in the calculation. However, only three heterozygous loci contributed to this average calculation. Such low PHR averages overall indicate that none of the WGA methods are amplifying the DNA in a representative fashion. Any bias introduced during the WGA process can then be further amplified by the STR PCR, resulting in DNA profile interpretation difficulties. Therefore these results indicate that further improvements must be made to the WGA process before this could be routinely introduced into LTDNA forensic casework.

4.3.2 Comparison of commercial and novel WGA kits

After the initial experiments, a novel WGA formulation, called the AT Kit, was provided by GE Healthcare for assessment and comparison to other commercial WGA kits. The second set of WGA experiments therefore aimed to determine the amplification efficiency of the AT Kit compared to the GenomiPhi V2 DNA Amplification Kit and the REPLI-g Mini Kit. Modifications to the recommended AT Kit protocol were also examined. The efficiency of these reactions was assessed through real time PCR quantification and multi-locus STR profiling as outlined below.

Quantification results for all samples can be seen in Figure 4.2. Results show that the AT Kit gave the highest levels of product after amplification when 10ng was used as the starting template. However below this amount, the AT Kit generally gave the lowest

quantification results. The REPLI-g kit, which gave the lowest quantification results for the 10ng samples, generally produced the highest quantification results for the low template samples. The exception to this was with the 50pg samples, which showed the highest amount of amplification product with the GenomiPhi kit followed by REPLI-g then the AT Kit. All samples were diluted to 500pg/µl based on the average of the triplicate quantification results for each sample, with 1µl of each dilution used as the starting template for multi-locus STR analysis using the PowerPlex® ESI 16 kit.

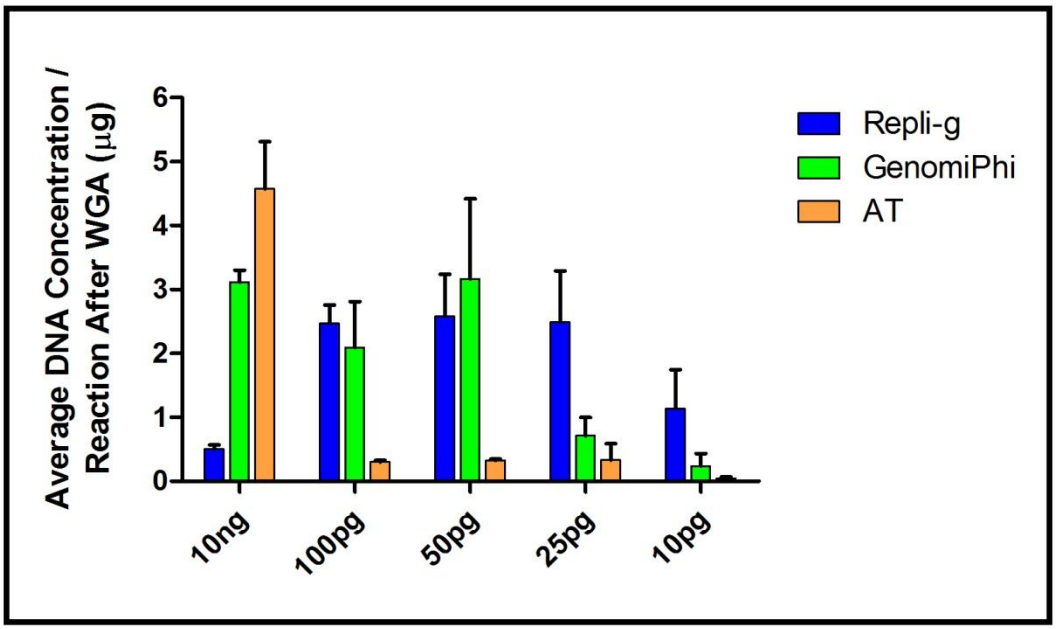

Figure 4.2 DNA quantification after whole genome amplification. Results represent the average DNA concentration result from five reactions for each WGA method with standard error of the mean.

Detailed STR results for control sample as well as samples amplified with the three WGA kits prior to STR analysis can be seen in Figure 4.3 and Tables 4.3 and 4.4. As expected STR analysis using 500pg genomic DNA starting template without prior WGA produced, profiles with 100% allele recovery. Heterozygous loci showed well balanced peaks with a PHR average of 82% and an average peak height of 2157 RFU. When the three WGA kits were used to amplify the recommended 10ng starting template all STR profiles from the amplified templates diluted to 500pg/µl displayed 100% allele recovery. Average PHRs for all samples amplified with the WGA kits

were slightly higher than the 500pg control samples. Average peak heights were similar for control and WGA samples. While 10ng genomic DNA is more than sufficient to perform STR analysis without prior WGA, these results could be useful for other techniques such as SNP genotyping by microarray or next generation sequencing that may require significantly higher amounts of starting template.

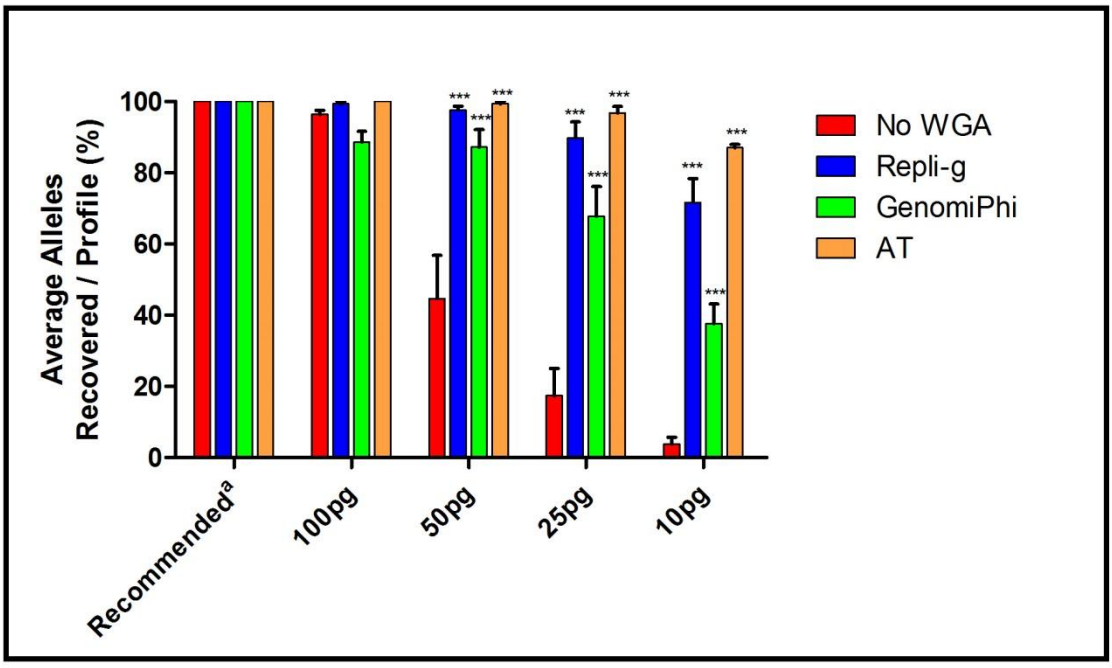

Figure 4.3 Correct alleles recovered in single source profiles of samples amplified with various commercial WGA kits. Results represent the average percentage of alleles recovered from five reactions for each WGA method. [a] For samples that underwent the various WGA procedures 10ng was used as the template for WGA. Since this amount too high to be used in an STR reaction, results were compared to STR profiles that used the ESI 16 kit recommended template of 500pg. All WGA samples were diluted to 500pg/µl for STR analysis. Error bars represent standard error of the mean. A 2-way ANOVA with Bonferroni post-tests was performed to compare samples amplified with the various WGA methods to samples amplified without prior WGA. As indicated, *** represents a p-value of less than 0.001.

Table 4.3 500pg/µl dilution of WGA products – allele recovery

		% Mean Correct Alleles Per Profile[a] (Std. Dev.)	% Allele Drop Out[b]	% Locus Drop Out[c]
Rec[d]:	Without WGA	100% (0)	0%	0%
	GenomiPhi	100% (0)	0%	0%
	REPLI-g	100% (0)	0%	0%
	AT	100% (0)	0%	0%
100pg:	Without WGA	96% (3%)	8%	0%
	GenomiPhi	88% (7%)	21%	1%
	REPLI-g	99% (1%)	1%	0%
	AT	100% (0%)	0%	0%
50pg:	Without WGA	45% (27%)	35%	38%
	GenomiPhi	87% (11%)	24%	1%
	REPLI-g	97% (3%)	5%	0%
	AT	99% (1%)	1%	0%
25pg:	Without WGA	17% (17%)	20%	73%
	GenomiPhi	68% (19%)	35%	15%
	REPLI-g	90% (10%)	20%	1%
	AT	97% (4%)	7%	0%
10pg:	Without WGA	4% (4%)	5%	94%
	GenomiPhi	37% (12%)	36%	45%
	REPLI-g	72% (15%)	33%	11%
	AT	87% (2%)	21%	3%

[a] Results represent average from five reactions for each WGA method
[b] Results represent the percentage of loci with ADO of 75 heterozygous loci
[c] Results represent the percentage of loci with LDO of 80 total loci
[d] Rec = Recommended template amount, with 10ng used as the starting template for all WGA reactions and 500pg used as the starting template for STR analysis without WGA.

Table 4.4 500pg/µl dilution of WGA products – peak heights and peak height ratios (PHR)

		Peak Heights			PHR - ADO (0%) Not Included			PHR - ADO (0%) Included		
		n[a]	Mean	Std. Deviation	n[b]	Mean	Std. Deviation	n[c]	Mean	Std. Deviation
Rec*[d]*:	Without WGA	155	2157	970	75	82%	13%	75	82%	13%
	GenomiPhi	155	2253	993	75	85%	13%	75	85%	13%
	REPLI-g	155	4179	2106	75	84%	11%	75	84%	11%
	AT	155	2869	1560	75	86%	9%	75	86%	9%
100pg:	Without WGA	149	290	129	69	70%	19%	75	65%	27%
	GenomiPhi	137	2550	2657	58	47%	29%	74	37%	32%
	REPLI-g	154	1717	1284	74	60%	25%	75	59%	25%
	AT	155	3376	2167	75	66%	20%	75	66%	20%
50pg:	Without WGA	69	157	54	19	76%	18%	45	32%	40%
	GenomiPhi	135	1385	1746	56	46%	27%	74	35%	30%
	REPLI-g	151	1880	2418	71	54%	24%	75	52%	27%
	AT	154	1168	1119	74	59%	23%	75	58%	24%
25pg:	Without WGA	27	146	39	5	77%	18%	20	19%	35%
	GenomiPhi	105	2164	2693	37	32%	28%	63	19%	26%
	REPLI-g	138	1908	2292	59	49%	28%	74	39%	32%
	AT	150	2700	2232	70	49%	25%	75	45%	27%
10pg:	Without WGA	6	132	35	1	85%	0%	5	17%	38%
	GenomiPhi	57	5249	3576	14	49%	43%	41	17%	34%
	REPLI-g	112	2433	2831	41	38%	29%	66	24%	29%
	AT	135	4657	3251	57	48%	32%	73	38%	35%

n[a] is the number of alleles recovered out of 155 total alleles per method

n[b] is the number of loci with both alleles present out of 75 total heterozygous loci

n[c] is the number of loci with at least one allele present out of 75 total heterozygous loci

[d] = Recommended template amount (1ng used as the starting template for all WGA reactions and 500pg used as the starting template for STR analysis without WGA).

As with the initial WGA experiments exaggerated stochastic variation was observed in all STR profiles from LTDNA without prior WGA, with allele and locus drop out increasing and peak heights decreasing with reduced starting template amounts. As expected, introducing any of the WGA procedures prior to STR analysis generally produced profiles with more correct alleles compared to control samples across most template amounts, with significant differences noted in all samples with starting templates of 50pg or less (Figure 4.3). No significant differences were seen in the allele recovery of the 100pg samples. With this starting template amounts both the AT kit and the REPLI-g kits produced slightly better allele recovery per profile compared to control samples (100% and 99% respectively compared to 96%), but the average allele recovery for the GenomiPhi amplified samples was only 88%. While this does not represent a significant reduction, it does indicate that even at this template amount the GenomiPhi kit is not amplifying all regions of the DNA equally, introducing a bias that may be further amplified with the STR reaction. This reduction in alleles seen with WGA samples compared to non-WGA controls has also been observed by others [115]. However in the work of Ballantyne et al [115], this phenomenon was mostly observed when a high starting template (1ng or 0.5ng) was used for WGA. Since reduced allele recovery was already observed with the 100pg samples, it was not surprising that the GenomiPhi kit consistently produced profiles with fewer alleles recovered compared to the other WGA methods with even lower starting template amounts. However with 50pg, 25pg and 10pg starting templates the allele recovery was still significantly increased with the GenomiPhi kit compared to STR profiles from control samples without WGA even if it was not as efficient as the other WGA methods.

The average allele recovery per STR profile was always highest with the AT Kit. This improvement was particularly notable in the 50pg, 25pg and 10pg samples where STR profiles from AT amplified samples showed an increase of 54%, 80% and 83% respectively in the average allele recovery per profile compared to control samples. Comparatively, the 50pg, 25pg and 10pg REPLI-g amplified samples showed an increase in the average allele recovery by 52%, 73% and 68% respectively compared to control samples. Results from this work show an improvement in allele recovery compared to other studies that examined the ability of WGA to amplify LTDNA for

forensic analysis [115, 125]. This could be due to the higher sensitivity STR kit used in this study compared to the other studies which used the AmpFlSTR® Profiler Plus® kit [115] and the AmpFlSTR® SGM Plus® kit [125].

Overall the PHR averages for the control samples without WGA were higher than the WGA samples when examining loci with both alleles present. As with the initial set of experiments, this is likely due to the increased allele recovery seen in the WGA samples. Small peaks not recovered in the control samples, and therefore not contributing to the PHR average, would be contributing to the PHR average in the WGA samples, resulting in lower PHRs for WGA samples. When 0% PHRs were included in the calculation, averages were generally higher for WGA samples compared to control samples. This is likely due to more loci showing both alleles with WGA resulting in fewer 0% scores contributing to the overall result.

Of the WGA methods, the AT amplified samples had the highest PHR average for most template amounts, indicating that the AT kit is amplifying the total DNA in a more representative fashion compared to the other kits. The exception was with the 10pg samples, where the highest PHR mean was seen with the GenomiPhi when only considering loci with both alleles present. As with the control samples, this is also likely due to the fact that the AT and REPLI-g methods allowed for additional smaller alleles to be detected that would reduce the PHR average, whereas these same alleles were not seen in the GenomiPhi samples, which showed the lowest allele recovery of the WGA methods. However, even though the AT amplified samples having the highest PHR averages of the WGA procedures, results for the 25pg and 10pg averages were still less than 50%, indicating that there is still preferential amplification between heterozygous alleles at some loci.

Despite triplicate real time PCR quantification of all WGA reactions, many of the profiles from LTDNA amplified samples – particularly from the 10pg samples

amplified with GenomiPhi or the AT kit – showed numerous over-amplified alleles and pull up peaks, indicating that there was more than the measured 500pg in each STR reaction. Such artefacts were not seen in the 10ng WGA samples. All WGA samples were quantified using primers targeting the OCA2 gene, located close to the centromere on chromosome 15q12 [171]. This gene was selected on the basis that it would give a more accurate quantification value since the WGA reaction has been shown to under represent the telomeric region of the hTERT gene used in the Quantifiler commercial qPCR kit [172]. However, since STR profiles demonstrate substantially more template has been used in the reaction than is indicated by the qPCR, it is likely that the GenomiPhi and AT kits are not amplifying the region of the OCA2 gene in the same fashion as the locations of the STRs examined, many of which are located towards the end of their respective chromosomes [173]. It would therefore be beneficial to test additional target genes for real time PCR quantification to determine which would give the most accurate qPCR result.

Since the STR results showed the WGA reactions were producing more DNA than the quantification results were indicating, all 100pg, 50pg, 25pg and 10pg WGA samples were diluted 1:100 for additional STR analysis. Results can be seen in Tables 4.5 and 4.6. Due to the increased dilution alleles were not over amplified, resulting in substantially reduced artefacts and pull up alleles seen in the STR profiles. However, this also had the effect of diluting out some of the actual alleles from the final profiles in the GenomiPhi and AT amplified samples, particularly at loci where the PHR was low in the 500pg dilution profiles. Further examination of different dilutions may therefore be beneficial to determine the most efficient level for WGA samples from low starting templates. Since the REPLI-g samples had the highest quantification results, diluting the samples 1:100 did not reduce the allele recovery results in the same manner as the GenomiPhi and AT kits. Indeed, the allele recovery was actually higher in the REPLI-g 1:100 dilutions compared to the 500pg dilution samples. Since the 500pg dilutions of the REPLI-g samples did not show the same level of artefacts as the GenomiPhi and AT amplified samples it would be preferable to maintain this dilution factor for the REPLI-g samples.

Table 4.5 1:100 dilution of WGA products – allele recovery

		% Mean Correct Alleles Per Profile[a] (Std. Dev.)	% Allele Drop Out[b]	% Locus Drop Out[c]
100pg:	GenomiPhi	92% (6%)	16%	0%
	REPLI-g	100% (0%)	0%	0%
	AT	97% (4%)	1%	1%
50pg:	GenomiPhi	84% (8%)	23%	5%
	REPLI-g	99% (2%)	3%	0%
	AT	90% (4%)	8%	6%
25pg:	GenomiPhi	54% (8%)	51%	22%
	REPLI-g	94% (2%)	13%	0%
	AT	74% (8%)	23%	15%
10pg:	GenomiPhi	24% (7%)	35%	60%
	REPLI-g	73% (8%)	37%	9%
	AT	63% (7%)	44%	18%

[a] Results represent the average of five reactions for each WGA method.
[b] Results represent the percentage of loci with ADO of 75 heterozygous loci
[c] Results represent the percentage of loci with LDO of 80 total loci

Table 4.6 1:100 dilution of WGA products – peak heights and peak height ratios (PHR)

		Peak Heights			PHR - ADO (0%) Not Included			PHR - ADO (0%) Included		
		n[a]	Mean	Std. Deviation	n[b]	Mean	Std. Deviation	n[c]	Mean	Std. Deviation
100pg	GenomiPhi	143	3540	2926	63	45%	31%	75	38%	33%
	REPLI-g	155	2415	1759	75	65%	27%	75	65%	27%
	AT	152	1150	965	73	66%	21%	74	65%	22%
50pg	GenomiPhi	130	1149	1687	54	39%	27%	71	30%	29%
	REPLI-g	153	2534	3009	73	53%	24%	75	51%	25%
	AT	139	587	523	64	56%	23%	70	51%	27%
25pg	GenomiPhi	84	1280	1746	21	31%	24%	59	11%	20%
	REPLI-g	145	2038	2079	65	46%	29%	75	40%	31%
	AT	114	512	471	46	48%	23%	63	35%	29%
10pg	GenomiPhi	37	1882	2431	5	43%	23%	31	7%	18%
	REPLI-g	113	1792	2265	40	36%	27%	68	21%	28%
	AT	97	1275	1637	31	47%	31%	63	23%	32%

n[a] is the number of alleles recovered out of 155 total alleles per method

n[b] is the number of loci with both alleles present out of 75 total heterozygous loci

n[c] is the number of loci with at least one allele present out of 75 total heterozygous loci

4.3.2.1 Modifications to the AT kit recommended protocol

Two modified methods – the Cycling protocol and the Split and Pool protocol – were tested on the AT Kit to determine if the allele balance and recovery could be improved compared to the standard method. Two high templates (10ng/µl, 1ng/µl), and two low template dilutions (100pg/µl and 10pg/µl) were generated and 1µl of each was used as the starting template for each modified AT reaction. For both modified procedures, three reactions were performed for each starting template amount.

All reactions were quantified using real time PCR. Using these values all samples were diluted to 500pg/µl for STR analysis. Results can be seen in Tables 4.7 and 4.8. For the 10ng and 1ng dilutions both modified methods produced profiles with 100% allele recovery. The Split and Pool protocol also produced 100% allele recovery per profile with 100pg starting template, the same result as the standard AT protocol.

The Split and Pool protocol showed similar PHRs and a slightly improved allele recovery per profile with 10pg starting template compared to both the Cycling and the standard AT protocols. This is surprising since each WGA aliquot would potentially contain less than one full genome copy, if the total DNA was divided equally between the reactions. It would therefore seem more likely that some regions of the DNA would be lost in this process. However, by splitting the reaction it may have allowed each small DNA amount to be equally represented in the amplification process. Without dividing the sample any early preferential amplification can be rapidly magnified by the fast processivity of the enzyme and hyperbranching of the final product.

The original proposition behind the Cycling method was that heating the reaction to 40°C would force the primers off the template, temporarily terminating the reaction. The amplification process could be then re-initiated at another random site on the template when the sample was cooled back to 30°C, to ultimately reduce any amplification bias that can occur in the WGA process. However, this process was not

successful since the Cycling protocol showed the lowest average allele recovery per profile and the lowest peak height ratio averages with 100pg and 10pg starting templates. One reason for this reduced allele recovery could be that the heating process worked to deteriorate the φ29 DNA polymerase activity. However, previous work has shown that enzymatic activity is maintained at 40°C [130] so this is unlikely. It could also be that an increase of the temperature to 40°C was insufficient to completely separate the newly formed strand from the original template so that the reaction could be reinitiated. Furthermore, even with the high processivity of the φ29 DNA polymerase, if the initial cycling process was successful, the DNA strands produced would only be approximately 400bp in length. While all of the STR loci in the ESI 16 kit produce fragments smaller than 400bp there is no guarantee that the new DNA produced by the WGA would encompass the STR loci entirely. As such the template would have similar results to degraded DNA, where allele and locus drop out are common despite having a high quantification result for the total template.

Table 4.7 Modified AT protocols – allele recovery

		% Mean Correct Alleles Per Profile[a] (Std. Dev.)	% Allele Drop Out[b]	% Locus Drop Out[c]
10ng:	Cycling	100% (0%)	0%	0%
	Split and Pool	100% (0%)	0%	0%
1ng:	Cycling	100% (0%)	0%	0%
	Split and Pool	100% (0%)	0%	0%
100pg:	Cycling	94% (4%)	11%	0%
	Split and Pool	100% (0%)	0%	0%
10pg:	Cycling	68% (11%)	27%	19%
	Split and Pool	90% (0%)	20%	0%

[a] Results represent the average of three reactions per modified AT method.
[b] Results represent the percentage of loci with ADO of 75 heterozygous loci
[c] Results represent the percentage of loci with LDO of 80 total loci

Table 4.8 Modified AT protocols – peak heights and peak height ratios (PHR)

		Peak Heights			PHR - ADO (0%) Not Included			PHR - ADO (0%) Included		
		n[a]	Mean	Std. Deviation	n[b]	Mean	Std. Deviation	n[c]	Mean	Std. Deviation
10ng	Cycling	93	1851	960	45	84%	13%	45	84%	13%
	Split and Pool	93	4939	2011	45	86%	10%	45	86%	10%
1ng	Cycling	93	1732	1188	45	75%	15%	45	75%	15%
	Split and Pool	93	4426	5101	45	86%	10%	45	86%	10%
100pg	Cycling	89	1541	1475	40	57%	24%	45	50%	29%
	Split and Pool	93	1958	1720	45	63%	22%	45	63%	22%
10pg	Cycling	64	3198	3287	25	27%	30%	37	18%	23%
	Split and Pool	84	4448	3378	36	43%	32%	45	34%	34%

n[a] is the number of alleles recovered out of 93 total alleles per method

n[b] is the number of loci with both alleles present out of 45 total heterozygous loci

n[c] is the number of loci with at least one allele present out of 45 total heterozygous loci

4.3.2.2 Mixture analysis using the AT kit

Two mixture samples were also examined with the AT kit using the standard protocol. The first mixture, "M1", contained DNA from two donors, both contributing low template levels of 50pg/µl for a total concentration of 100pg/µl. The second mixture, "M2", also contained DNA from two donors, but in this mixture one donor contributed the majority of the template of 450pg/µl while the other contributed a low template amount of 50pg/µl for a total concentration of 500pg/µl. Five AT reactions were performed for each mixture. Each AT reaction was quantified using real time PCR and a 500pg/µl dilution was made from each sample for STR analysis. Five STR reactions were also performed on 1µl of the original mixture samples without prior WGA. Figure 4.4 shows the total allele recovery per profile from both contributors, while Figure 4.5 shows the average alleles recovered in each profile from the individual contributors.

Without prior WGA, STR profiles of M1 showed an average of 65% of the total alleles from both contributors recovered. When the AT reactions were performed the average allele recovery per profile increased to 81%. This average would have been higher but one of the AT reactions failed, resulting in only 7 of the total 53 alleles (13%) being recovered in the STR profile. The median percentage alleles recovered per profile for the AT amplified M1 samples was actually 96%. However, while allele recovery was greater with prior AT amplification, mixture profiles were not resolvable since the mixture contained an equal amount of DNA from both contributors and this 1:1 balance was maintained through the AT process.

The STR profiles from M2 samples with and without AT amplification showed 100% of the alleles from the major contributor in all profiles. The average allele recovery per profile from the minor contributor was 80% without AT amplification. With AT amplification this was reduced to 71%. This is likely due to the abundance of the major contributor DNA in the sample compared to the minor contributor, resulting in preferential amplification of the major contributor.

This highlights one of the major difficulties of working with mixture samples. Even if the amount of DNA present in a sample from each contributor could be quantified, there is no method for separating the DNA from individual contributors. Therefore only the maximum amount of DNA can be added to the STR reaction regardless of the quantification from each contributor otherwise amplification artefacts may begin to appear in the profiles. Since these results indicate that WGA only serves to over-amplify the major component of a mixed sample, this prior amplification may not be the best option for analysing samples with unequal contributions from donors.

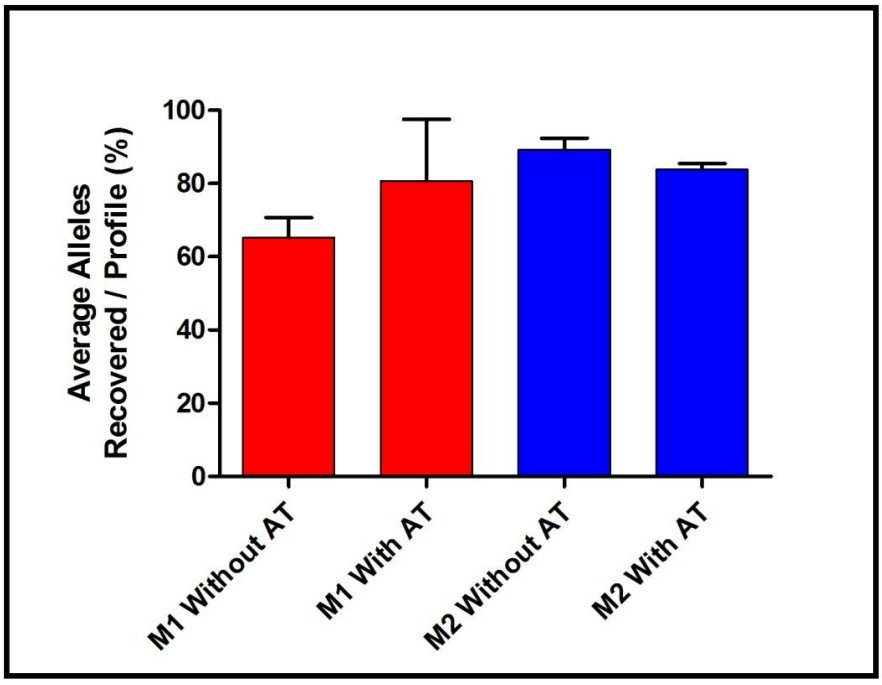

Figure 4.4 Total alleles recovered in mixture profiles. Results represent the average percentage alleles recovered in each profile from a possible 53 total alleles. Five reactions were performed for each method. Error bars represent standard error of the mean. Unpaired 2-tailed T-tests were performed to compare samples amplified with and without AT amplification. No significant differences were found.

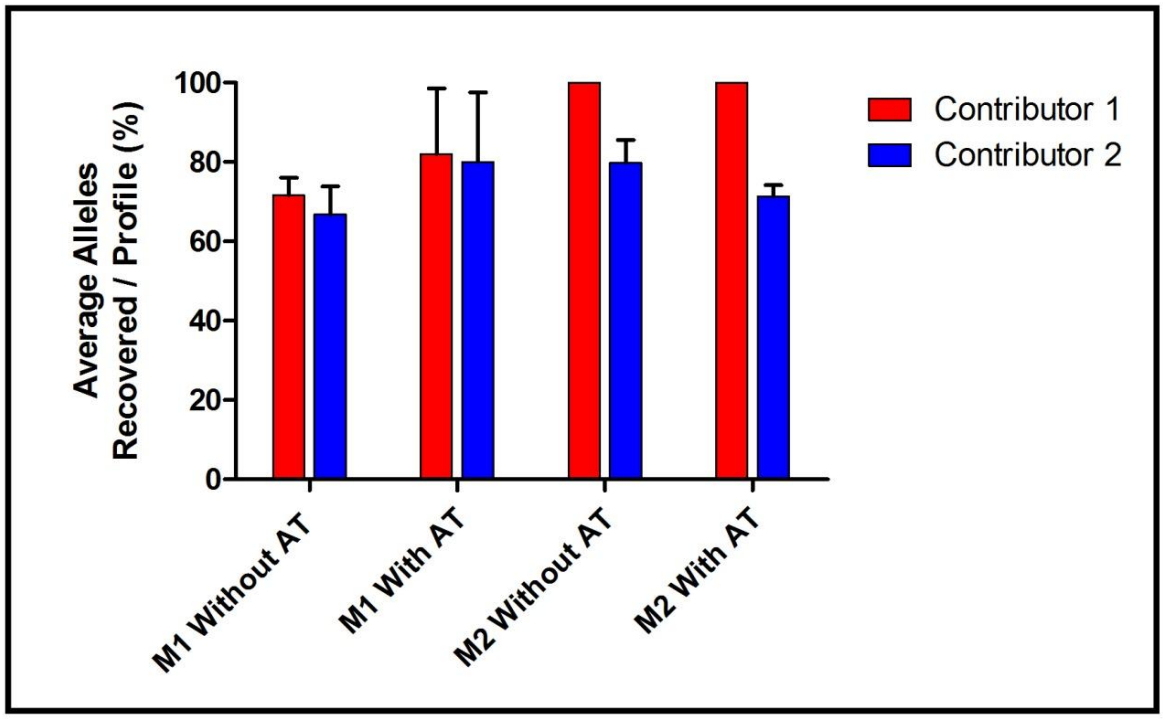

Figure 4.5 Alleles recovered from each contributor in mixture profiles. Results represent the average percentage alleles recovered in each profile from a possible 31 alleles for Contributor 1 and 30 alleles for Contributor 2. Five reactions were performed for each method. Error bars represent standard error of the mean.

4.4 Conclusion

Overall the AT kit was the most effective of the examined kits to amplify single source low template DNA samples. STR analysis of the WGA products showed allele recovery and peak height ratios were highest when LTDNA samples were subjected to the AT kit, followed by the REPLI-g kit then the GenomiPhi kit. However, all three chemistries allowed for significantly more alleles to be recovered compared to LTDNA samples that did not undergo prior processing when the optimum amount of WGA template was used for STR analysis.

Despite having the best results of the three kits, drop out still occurred with the AT kit when less than 50pg of DNA was used as the starting template and significant allele

imbalance was seen in some loci. Such stochastic variation can make STR profile interpretation difficult, and therefore further improvement is needed before this WGA procedure could be routinely implemented into the forensic DNA analysis workflow for casework.

Modifications to the AT protocol showed varying results. The Cycling protocol samples consistently showed reduced allele recovery and PHRs compared the standard protocol. However, the Split and Pool Protocol showed equal or slightly higher allele recovery and similar PHRs compared to the standard protocol. The slight increase in allele recovery with 10pg starting template is promising, suggesting that dividing the reaction allows for the small amount of template in each aliquot to be amplified in a more representative fashion than would be if the reaction was kept whole for amplification. However, like with the standard protocol samples, since stochastic variation was present in the Split and Pool WGA STR profiles - even if it was at reduced levels - further improvements are necessary before implementation into routine casework.

WGA of mixture samples using the AT kit showed an increase in alleles recovered when both contributors were at equal low template levels. However, when the mixture samples contained unequal contributions from donors, the WGA reaction preferentially amplified the major profile resulting in a reduced number of minor profile alleles recovered compared to samples that did not undergo prior WGA. This indicates that while WGA may be useful for single source samples or low template mixed samples where donors contribute equal amounts of DNA to the sample, it is not the preferred analysis option for mixtures with major and minor contributors.

CHAPTER 5

LOW COPY ANALYSIS USING INTACT CELLS

5.1 Introduction

Laser microdissection (LMD) can be used to identify and isolate single or small numbers of cells from cell populations [78-83]. This technique could be particularly useful for forensic DNA analysis. Mixtures of body fluids from different individuals are common in forensic casework and the ability to separate cells from individual contributors would be beneficial [79]. Samples with limited numbers of cells are also becoming an increasingly analysed type of forensic evidence, therefore specific LCN techniques that deal with these sample types are necessary [83]. DNA profiling from limited template samples could be improved if all possible genetic material was able to be collected. The use of isolated cells allows for a known number of genome copies to be analysed in each reaction. The decision to apply LCN techniques relies on quantification results of the genomic DNA. However, such results may not be accurate if the sample contains a very low level of DNA. Furthermore, quantification results do not guarantee that an equal number of both alleles are contained in the sample. The use of whole cells overcomes such limitations.

Forensic STR analysis has been performed on laser microdissected cells, with full DNA profiles obtained from a minimum of 15 epithelial cells or 30 sperm cells using the AmpFlSTR® Identifiler® PCR Amplification Kit (Life Technologies, Carlsbad, CA, USA) with 28 PCR cycles [88]. However, these results were not consistent and it was recommended that at least 50 epithelial cells or 150 sperm cells be collected for optimum DNA profiling results [88]. Such cell numbers would be the equivalent of approximately 0.3ng or 0.45ng genomic DNA for buccal and sperm cells respectively, less than half of the recommended starting template for the Identifiler® kit, which has a recommend DNA input of 1ng genomic DNA **[**174**]**. Higher sensitivity STR kits, such as the PowerPlex® ESI 16 Kit (Promega Corp, Madison, WI, USA), have since been developed that require less starting DNA (0.5ng) [173]. Such kits could potentially be beneficial for the analysis of single or small numbers of isolated cells since less starting DNA template is required.

Further improvements in the analysis of limited numbers of cells could also be achieved by applying WGA techniques. Some success has been obtained with the use of MDA on single or small numbers of cells [119-123]. However, in these studies cell samples were isolated by mouth pipetting into small volumes of less than 5µl [119, 122, 123] or diluted to approximate cell counts [121]. Such small volumes are not possible with LMD systems that use the gravity method of collection as sufficient liquid (approximately 8µl) must be placed in the collection cap to will cover the entire surface so that cells remain in place once isolated [88].

In this chapter single or small numbers of LMD cells were used as template for forensic STR analysis. Cell samples were amplified using both standard and increased cycle protocols to determine if improved results could be obtained. Cells were also subjected to WGA prior to STR analysis to determine the compatibility of LMD and WGA. Results described in the previous chapter (see figure 4.3) showed that the novel AT Kit from GE Healthcare gave the best results for low template DNA. Therefore, this kit was used to amplify the LMD cell samples for downstream STR profiling. Various DNA extraction procedures were examined including the One-Tube method [88], heat denaturation, spin columns and a modified alkaline lysis procedure, to determine which, if any, were the most effective for use with AT Kit.

5.2 Methods

5.2.1 Sample preparation

This project was approved by the Bond University Human Research Ethics Committee (BUHREC), approval number RO743. Four buccal cell samples were collected from an anonymous donor with informed consent using a cotton swab and allowed to air dry. For three of the swabs, the cotton tip was removed and immersed in 200ul of PBS in a sterile 2ml tube. The tube was vortexed to allow the release of cells from the cotton tip. The tip was squeezed and removed using sterile tweezers. The remaining cotton swab was kept as a control sample.

5.2.2 Slide preparation

Slides were prepared according to an internally developed staining method used by the Victorian Police. In this method 3 x 10ul aliquots of the buccal cells in PBS were placed on a polyethylene naphthalate (PEN) membrane slide (Leica Microsystems, Wetzlar, Germany) and dried for 15 minutes on a 34°C heat block. Slides were then removed from the heat block and left to cool at room temperature for 15 minutes. To stain the slide 2-3 drops of haematoxylin were placed onto the slide and left for 1 minute. Scott's Tap Water (Leica Microsystems) was added to the slide for 30 seconds to rinse off the haematoxylin. 2-3 drops of eosin were placed on the slide for 10 seconds, which was then rinsed with 70% ethanol. The slide was placed in a 70% ethanol bath for 5 minutes and then allowed to air dry at room temperature for 10 minutes.

5.2.3 Laser microdissection

Prepared slides were viewed using a Leica LMD 6500 Laser Microdissection System (Leica Microsystems) at 20x magnification to identify and mark cells for collection. Groups of 50, 10, 5, 2 or 1 buccal cells were isolated by laser ablation and collected in the lid of a sterile 0.2ml PCR tube containing either 8µl of 1x TE buffer (10mM Tris/1mM EDTA) or 8µl of Extraction Buffer (EB) containing 7µl 1x TE and 1µl Tween 20 (1%) as described by Meredith et al [88]. Tube lids were viewed under 5x magnification to ensure cells had been collected.

5.2.4 DNA extraction

DNA was extracted from the remaining buccal swab using the BioRobot EZ1® Workstation with the EZ1® DNA Tissue Kits (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Cells collected in EB were extracted using the "One-Tube Extraction Method" described by Meredith et al. [88]. Four different DNA extraction methods were examined for cells collected in TE buffer: 1) the One-Tube method; 2) heat denaturation; 3) QIAamp® DNA Micro spin columns (QIAGEN, Hilden, Germany); 4) a modified alkaline lysis procedure provided by GE Healthcare.

5.2.4.1 One-Tube extraction method

In the One-Tube Extraction Method, 0.2ml tubes containing isolated cells were centrifuged for 1 minute at 13000 RPM to ensure all LMD cells were recovered from the PCR tube cap. Cells were then gently resuspended and 1µl of 1mg/ml Proteinase K added to each tube. Samples were incubated for 1 hour at 56°C followed by 10 minutes at 95°C then held at 4°C for WGA or STR analysis.

5.2.4.2 Heat denaturation

For the heat denaturation, samples were incubated at 99°C for 10 minutes then cooled to 4°C for WGA analysis.

5.2.4.3 QIAamp® DNA Micro spin columns

QIAamp® DNA Micro spin columns were used according to the manufacturer's instructions using the "Isolation of Genomic DNA from Laser-Microdissected Tissues" protocol contained in the QIAamp® DNA Micro Handbook.

5.2.4.4 Modified Alkaline Lysis procedure

In the modified alkaline lysis procedure, 1ul of 1.6 M KOH was added to each tube to lyse the cells. The samples were then frozen at -80C for 1 hour, followed by a 10 minute incubation at 65°C. 1ul of 1.6 M HCl/0.6 M Tris, pH 7.5 was added to neutralise the reaction and samples were cooled to 4°C.

5.2.5 Whole genome amplification

WGA was performed on the entire cell sample extraction using the novel AT Kit (GE Healthcare) using methods described in Chapter 4. However the reaction volume was doubled to 40ul to accommodate the volume of the cell samples. After WGA, the amount of product was quantified in triplicate using the method described in section

2.2.1. Samples were diluted to 500pg/µl for STR analysis. If the quantification result was less than 500pg/µl, 1µl of the neat WGA sample was used for STR analysis.

5.2.6 Short tandem repeat analysis

STR analysis was performed on the entire 9µl of each extracted cell, or 1ul of the diluted WGA reaction using the PowerPlex® ESI 16 Kits (Promega Corp, Madison, WI, USA) according to the manufacturer's instructions. The cells that did not undergo WGA were amplified with either 30 or 34 PCR cycles. Profiles were also obtained for 6 replicates of each 6pg, 12pg, 30pg and 60pg dilutions, the equivalent amount of DNA that should be in the 1-, 2-, 5- and 10-cell samples respectively, using 30 and 34 PCR cycles for comparison.

Electropherograms for all samples were obtained using the 3130 Genetic Analyser (Life Technologies). For each sample a loading cocktail of 10µl Hi-Di™ Formamide (Life Technologies) and 1µl of CC5 Internal Lane Standard 500 (Promega Corp) was mixed with 1µl of amplified product and denatured for three minutes at 95°C. After cooling, samples were injected on the 3130 using a 3kv, 5 second injection as is the recommended PowerPlex® ESI 16 protocol. Data were analysed using Genemapper ID® software version 3.2.1 (Life Technologies) and PowerPlex® ESI 16 panels and bins files. A detection threshold of 50 RFU was used for analysis of all sample profiles.

All electropherograms were compared to a reference profile to determine allele drop out (ADO) and locus drop out (LDO). Peak heights and peak height ratios (PHR) were also recorded. PHRs were determined by dividing the height of the smaller peak in a heterozygote pair by the height of the larger peak. PHR averages were calculated with and without ADO included.

5.3 Results and Discussion

5.3.1 Laser microdissection

Buccal cells were isolated by LMD in groups of 1, 2, 5, 10 or 50 cells. A total of 25 of each cell number were collected, 10 of which were collected in EB and 15 in TE. PEN slides were viewed at 20x magnification for identification, selection and isolated of buccal cells. All tube caps were viewed at 5x magnification to ensure cells were collected (Figures 5.1 to 5.5).

5.3.2 Standard and increased cycle STR analysis of LMD cells

Six of each 1-, 2-, 5-, 10-, and 50-cell samples were extracted using the One-Tube method and amplified using the PowerPlex® ESI 16 kit recommended 30-cycle protocol. All 50-cell samples showed the complete STR profile. This is expected since each 50-cell sample should contain approximately 300pg genomic DNA. Stochastic variation was observed in most STR profiles obtained using 10 or fewer cells as the starting template, with allele and locus drop out increasing and peak heights decreasing with reduced cell numbers (Table 5.1). Of the 10-cell samples, two gave complete STR profiles while the remaining four gave partial profiles, resulting in an average of 80% correct alleles recovered per profile. Below this cell number all profiles showed partial results only, with all displaying 50% or less of the correct alleles.

Complete amplification failure was evident in some samples with 5 cells or less, with zero alleles recovered in one 5-cell samples, two 2-cell samples and all 1-cell samples. In studies conducted by others, at least 15 epithelial cells were necessary to obtain a complete STR profile using the AmpFlSTR® Identifiler® PCR Amplification Kit [88]. Results presented here show an increase in allele recovery with the use of a higher sensitivity STR kit. However, improvements are still necessary due to the high allele and locus drop out observed in most STR profiles.

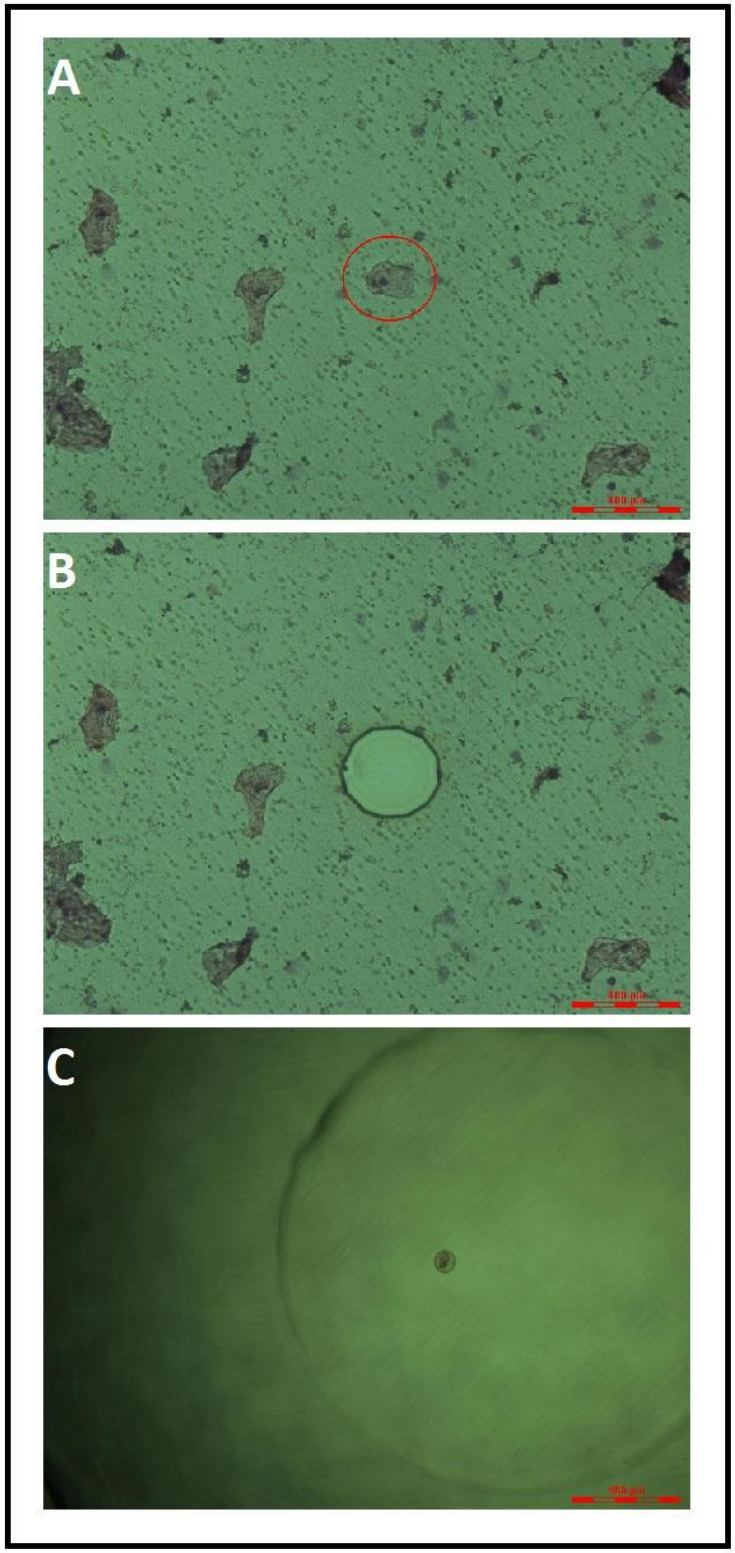

Figure 5.1 Laser microdissection of one cell. Selection and isolation of a single buccal cell, where A shows the PEN slide before collection, B shows the PEN slide after collection (both 20x magnification) and C shows the cap containing the isolated cell (5x magnification).

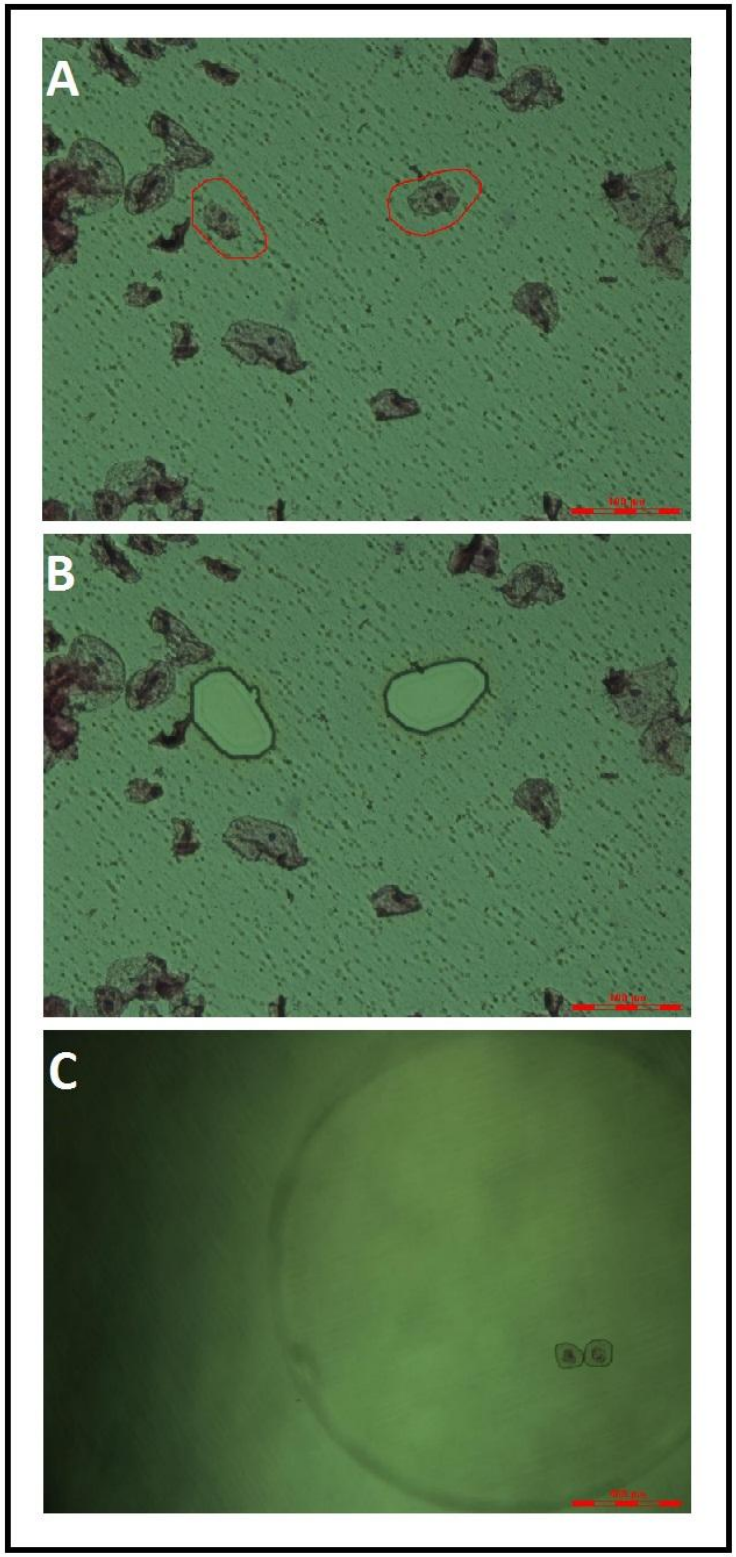

Figure 5.2 Laser microdissection of two cells. Selection and isolation of two buccal cells, where A shows the PEN slide before collection, B shows the PEN slide after collection (both 20x magnification) and C shows the cap containing the isolated cells (5x magnification).

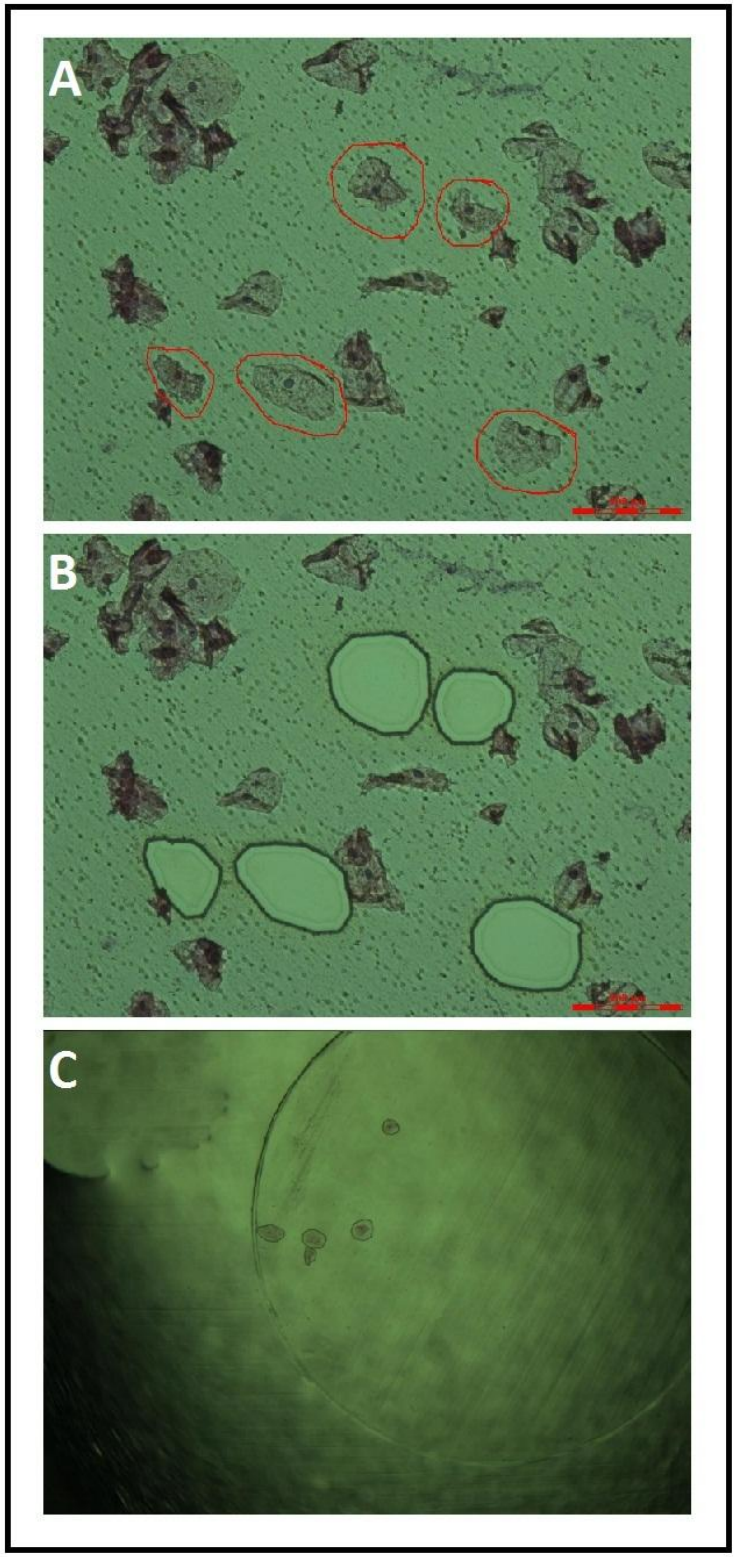

Figure 5.3 Laser microdissection of five cells. Selection and isolation of five buccal cells, where A shows the PEN slide before collection, B shows the PEN slide after collection (both 20x magnification) and C shows the cap containing the isolated cells (5x magnification).

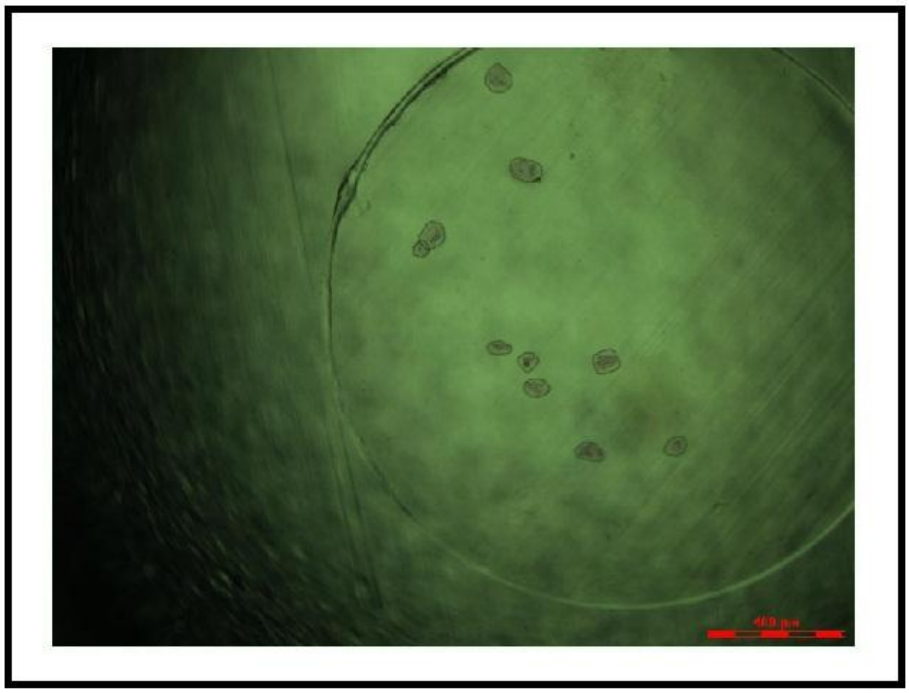

Figure 5.4 Laser microdissection of ten cells. Collection cap containing ten isolated buccal cells at 5x magnification.

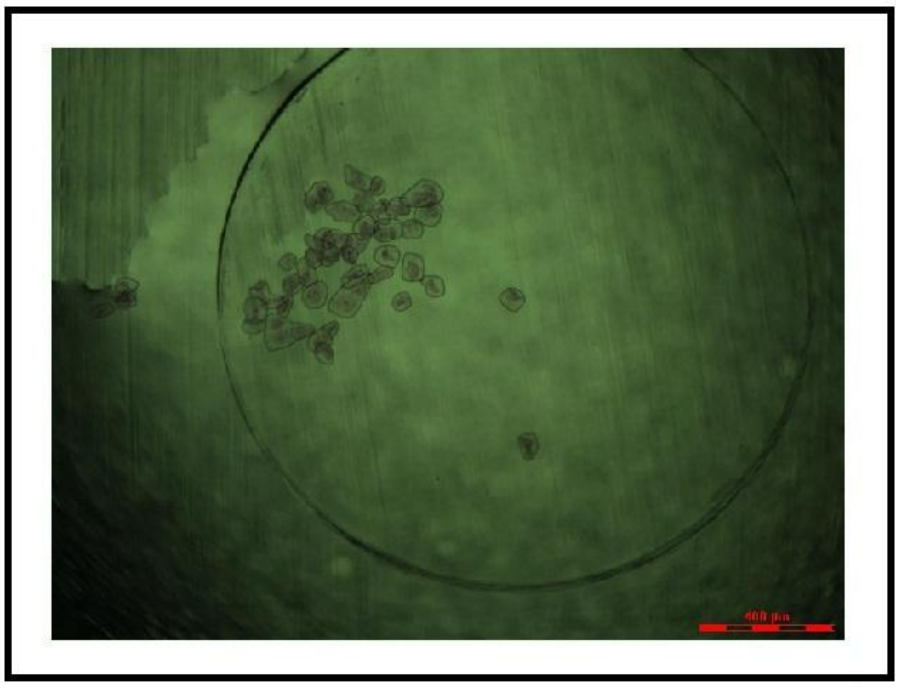

Figure 5.5 Laser microdissection of fifty cells. Collection cap containing fifty isolated buccal cells at 5x magnification.

Table 5.1 Allele recovery and balance from LMD cells amplified with 30 PCR cycles

Number of cells	% Mean Correct Alleles Per Profile[a] (SD)	% Allele Drop Out[b]	% Locus Drop Out[c]	Peak Heights			PHR - ADO (0%) Not Included			PHR - ADO (0%) Included		
				n[d]	Mean	Std. Deviation	n[e]	Mean	Std. Deviation	n[f]	Mean	Std. Deviation
50	100% (0%)	0%	0%	138	622	400	42	78%	12%	42	78%	12%
10	80% (27%)	26%	11%	110	133	83	25	69%	17%	36	48%	35%
5	18% (16%)	14%	75%	25	88	47	1	83%	0%	7	12%	31%
2	17% (19%)	10%	79%	23	69	18	3	85%	16%	7	36%	46%
1	0% (0%)	0%	100%	0	-	-	0	-	-	0	-	-

[a] N = 6 for each cell number

[b] The percentage of loci with ADO of 42 heterozygous loci

[c] The percentage of loci with LDO of 96 total loci

[d] The number of alleles recovered out of 138 total alleles per method

[e] The number of loci with both alleles present out of 42 total heterozygous loci

[f] The number of loci with at least one allele present out of 42 total heterozygous loci

Additional alleles were observed when the cell samples were subjected to an increased cycle PCR (Figure 5.6). The results are shown in Table 5.2. When amplified with a 34-cycle reaction, half the 10-cell samples gave complete STR profiles. The other half showed either one or two loci with ADO. Overall, an average of 96% of the correct alleles was recovered per profile which is an increase of 17% compared to the 30 cycle 10-cell samples. One of the 5-cell samples also gave a complete STR profile. Of the remaining 5-cell samples, 4 showed partial profiles with more than 50% of the correct alleles recovered. However in one profile only a single allele was recovered. On average, 69% of the correct alleles were recovered in each 5-cell profile, an increase of 51% compared to 30 cycle samples. The 2- and 1-cell samples also showed increased allele recovery with 34 PCR cycles compared to the samples amplified with the recommended 30 cycles. However, the majority of samples showed less than 50% of the correct alleles, with an average allele recovery per profile of 35% for the 2-cell samples and 28% for the 1-cell samples. Complete amplification failure occurred in one 2-cell sample and one 1-cell sample.

PHRs were generally not affected by the increase in PCR cycles when examining loci with both alleles present (Figure 5.7). PHR averages were similar across different cell amounts, for samples amplified with 30 and 34 cycles. The exception to this was with the 1-cell samples, which showed a significant difference in the PHR average of the samples amplified with 34 cycles since no alleles were recovered and thus no PHRs recorded for samples amplified with 30 PCR cycles. When 0% results were included in the calculation, mean PHRs were generally higher for samples amplified with 34 cycles compared to samples amplified with 30 cycles. This is likely due to the increased allele recovery seen in samples amplified with 34 cycles so that there are less 0% results contributing to the mean compared to samples amplified with 30 PCR cycles. Compared to the samples amplified with 30 PCR cycles, the mean peak height for each of the cell numbers was improved when amplified with 34 cycles. Overall these results indicate that, despite the improved allele recovery, increasing the PCR cycles alone is not sufficient to consistently recover complete and balanced STR profiles from samples with limited cell numbers.

Table 5.2 Allele recovery and balance from LMD cells amplified with 34 PCR cycles

Number of cells	% Mean Correct Alleles Per Profile[a] (SD)	% Allele Drop Out[b]	% Locus Drop Out[c]	Peak Heights			PHR - ADO (0%) Not Included			PHR - ADO (0%) Included		
				n[d]	Mean	Std. Deviation	n[e]	Mean	Std. Deviation	n[f]	Mean	Std. Deviation
10	97% (4%)	10%	0%	134	897	834	38	70%	17%	42	63%	26%
5	69% (30%)	24%	23%	95	480	403	21	69%	29%	31	47%	40%
2	35% (24%)	29%	54%	48	247	195	4	73%	20%	16	18%	34%
1	28% (28%)	24%	63%	38	318	269	3	44%	9%	13	10%	20%

[a] N = 6 for each cell number

[b] The percentage of loci with ADO of 42 heterozygous loci

[c] The percentage of loci with LDO of 96 total loci

[d] The number of alleles recovered out of 138 total alleles per method

[e] The number of loci with both alleles present out of 42 total heterozygous loci

[f] The number of loci with at least one allele present out of 42 total heterozygous loci

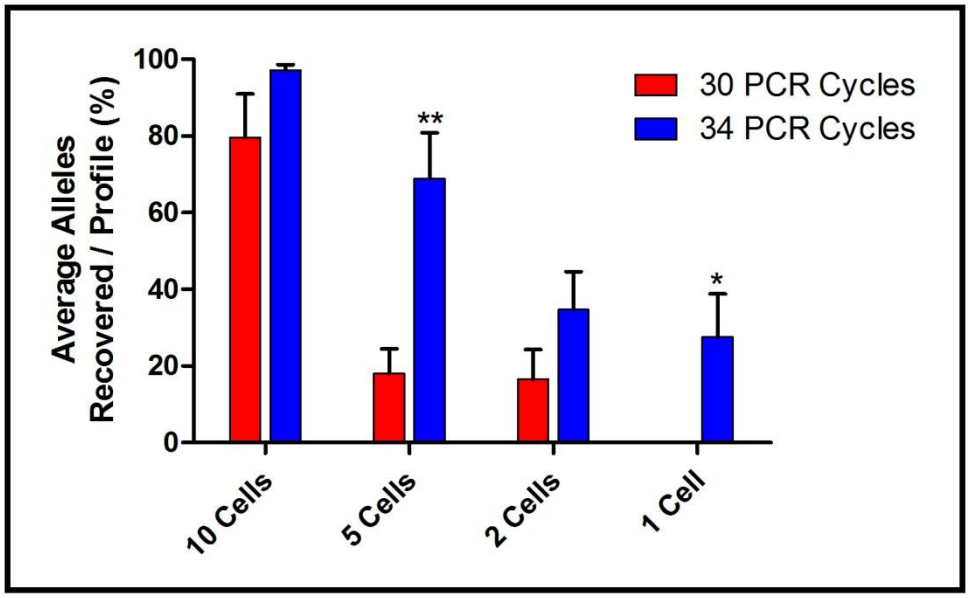

Figure 5.6 Correct alleles recovered in profiles of LMD cell samples amplified with 30 and 34 PCR cycles. Results represent the average percentage of alleles recovered from six reactions for each cell number and amplification method. Error bars represent standard error of the mean. Unpaired, 2-tailed T-Tests were performed to compare results from samples amplified with 30 PCR cycles to samples amplified with 34 PCR cycles. As indicated,* represents a p-value of less than 0.05, and ** represents a p-value of less than 0.01.

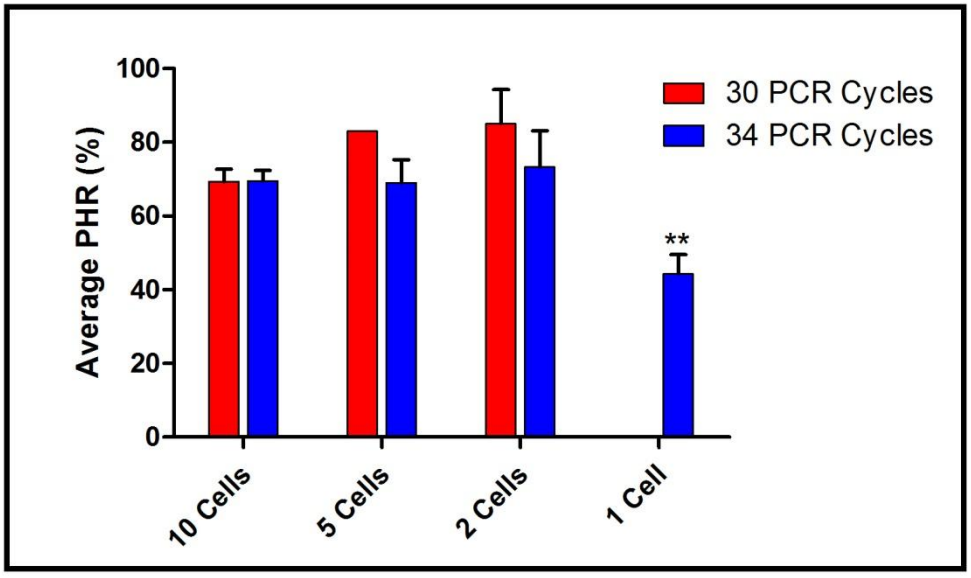

Figure 5.7 Peak height ratios in profiles of LMD cell samples amplified with 30 and 34 PCR cycles. Results represent the average PHR from all heterozygous loci where both alleles were recovered from six reactions for each cell number and amplification method. Error bars represent standard error of the mean. Unpaired, 2-tailed T-Tests were performed to compare results from samples amplified with 30 PCR cycles to samples amplified with 34 PCR cycles. As indicated, ** represents a p-value of less than 0.01.

5.3.3 Standard and increased cycle STR analysis of cell-equivalent DNA samples

STR profiles were also obtained for 6 replicates of each 60pg/µl, 30pg/µl, 12pg/µl and 6pg/µl dilutions, the equivalent amount of DNA that should be in the 10-, 5-, 2- and 1-cell samples respectively, using 30 and 34 PCR cycles (Tables 5.3 and 5.4). This was done to determine if the cell samples were amplifying in the same manner as DNA dilutions or if components of the cells or chemicals involved in the extraction were limiting the success of the reaction. As seen in Figures 5.8 and 5.9, allele recovery was higher in DNA dilutions across all template amounts with 30 and 34 PCR cycles compared to cell samples. The greatest difference was seen in the comparison of 5 cell samples and the 30pg samples amplified with 30 PCR cycles, which shows an increase of 47% in the average alleles recovered per profile for the DNA dilution compared to the whole cell sample. While this may indicate a potential inhibitor in the cell samples or extraction chemicals, it may also indicate a potential issue with the qPCR of genomic DNA. DNA dilutions may contain more genomic material than the cell samples if the quantification result for the original DNA sample was not precise. Since the number of cells in each LMD sample is precisely known, it could be that DNA dilutions are not faithfully reflecting the results that can be realistically achieved from that exact amount of genetic material obtained directly from the cells.

Table 5.3 Allele recovery and balance from cell-equivalent DNA samples amplified with 30 PCR cycles

	% Mean Correct	% Allele	% Locus	Peak Heights			PHR - ADO (0%) Not Included			PHR - ADO (0%) Included		
	Alleles Per Profile[a] (SD)	Drop Out[b]	Drop Out[c]	n[d]	Mean	Std. Deviation	n[e]	Mean	Std. Deviation	n[f]	Mean	Std. Deviation
60pg	90% (4%)	9%	5%	168	167	89	77	69%	19%	85	63%	27%
30pg	65% (11%)	34%	18%	121	112	59	42	67%	19%	73	39%	37%
12pg	28% (8%)	30%	57%	52	82	36	11	73%	16%	38	21%	34%
6pg	10% (5%)	17%	82%	18	63	14	1	80%	0%	16	5%	20%

[a] N = 6 for each cell number

[b] The percentage of loci with ADO of 90 heterozygous loci

[c] The percentage of loci with LDO of 96 total loci

[d] The number of alleles recovered out of 186 total alleles per method

[e] The number of loci with both alleles present out of 90 total heterozygous loci

[f] The number of loci with at least one allele present out of 90 total heterozygous loci

Table 5.4 Allele recovery and balance from cell-equivalent DNA samples amplified with 34 PCR cycles

	% Mean Correct	% Allele	% Locus	Peak Heights			PHR - ADO (0%) Not Included			PHR - ADO (0%) Included		
	Alleles Per Profile[a] (SD)	Drop Out[b]	Drop Out[c]	n[d]	Mean	Std. Deviation	n[e]	Mean	Std. Deviation	n[f]	Mean	Std. Deviation
60pg	97% (4%)	0%	2%	143	1133	1140	49	72%	23%	49	72%	23%
30pg	91% (10%)	8%	5%	136	777	781	44	54%	25%	48	49%	28%
12pg	70% (9%)	37%	17%	105	438	473	24	53%	21%	43	30%	31%
6pg	47% (11%)	31%	43%	70	339	355	15	64%	18%	31	31%	35%

[a] N = 6 for each cell number

[b] The percentage of loci with ADO of 51 heterozygous loci

[c] The percentage of loci with LDO of 96 total loci

[d] The number of alleles recovered out of 147 total alleles per method

[e] The number of loci with both alleles present out of 51 total heterozygous loci

[f] The number of loci with at least one allele present out of 51 total heterozygous loci

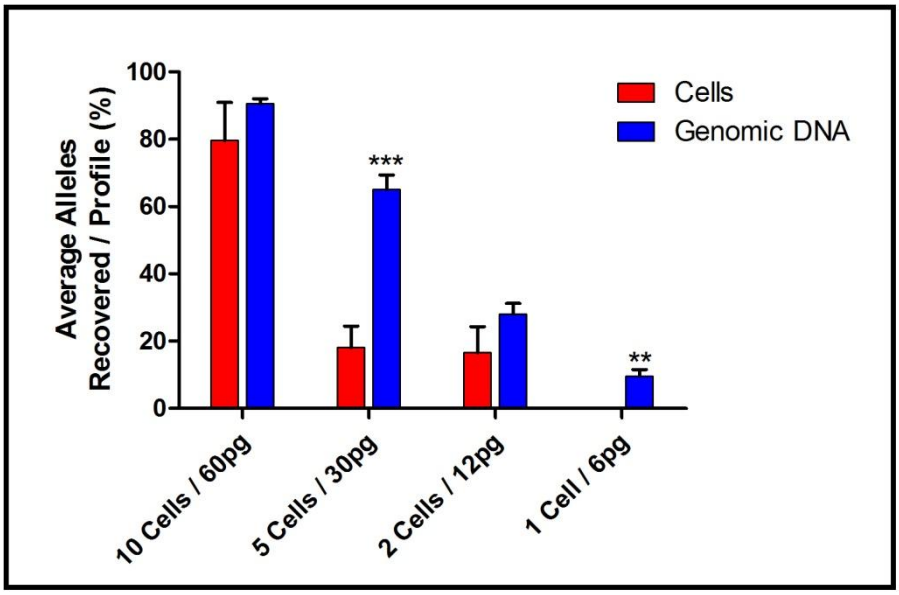

Figure 5.8 Correct alleles recovered in profiles of LMD cell samples and equivalent amounts of genomic DNA amplified with 30 PCR cycles. Results represent the average percentage of alleles recovered from six reactions for each cell number and DNA amount. Error bars represent standard error of the mean. Unpaired, 2-tailed T-Tests were performed to compare results from cell and genomic DNA samples. As indicated,** represents a p-value of less than 0.01, and *** represents a p-value of less than 0.001.

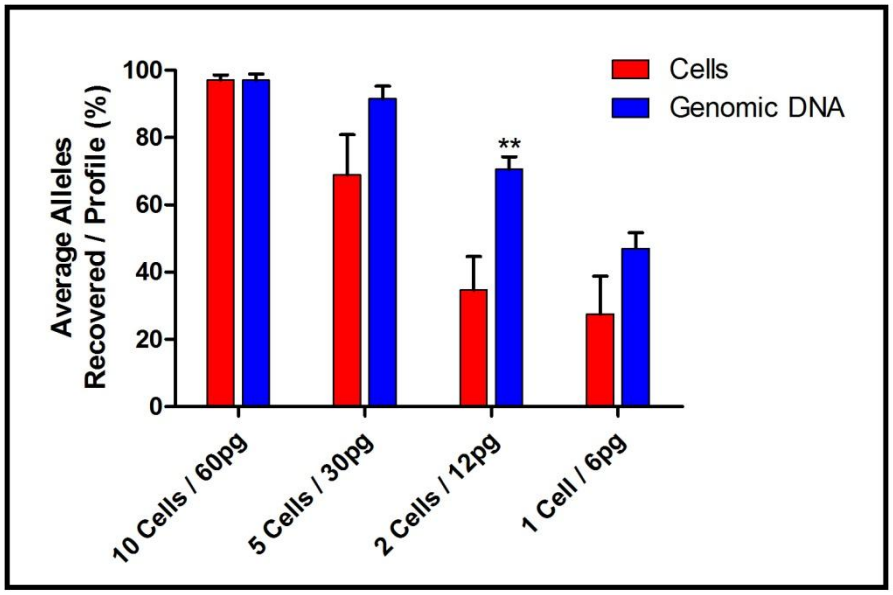

Figure 5.9 Correct alleles recovered in profiles of LMD cell samples and equivalent amounts of genomic DNA amplified with 34 PCR cycles. Results represent the average percentage of alleles recovered from six reactions for each cell number and DNA amount. Error bars represent standard error of the mean. Unpaired, 2-tailed T-Tests were performed to compare results from cell and genomic DNA samples. As indicated,** represents a p-value of less than 0.01.

5.3.4 Whole genome amplification with One-Tube extraction

Results from Chapter 4 showed that improved DNA profiles could be obtained from low template samples that underwent WGA using the AT Kit prior to STR analysis. As such, this technique was applied to the LMD cell samples to determine if further alleles could be recovered compared to STR typing alone. Three of each 1-, 2-, 5-, 10- and 50-cell samples were extracted using the One-Tube method, and the entire extract was used as template for the AT reaction. However, qPCR quantification results of the AT product show that amplification was largely unsuccessful. As seen in Table 5.5, while some of the samples showed a slightly higher quantification compared to the original starting template amount, most of the samples appeared to contain less DNA after AT amplification. Furthermore, qPCR results from one of each of the 1-, 2- and 5-cell samples showed no amplifiable product. STR analysis was performed on AT extracts to determine if results could still be obtained despite the low quantification results. As expected none of the samples produced successful STR results. These results indicated that the One-Tube extraction method is likely not compatible with the WGA reaction and as such alternative extraction methods were investigated.

5.3.5 Whole genome amplification with alkaline lysis extraction

Three of each 1-, 2-, 5-, 10- and 50-cell samples were extracted using a modified alkaline lysis procedure provided by GE Healthcare. The alkaline lysis method is recommended by GE Healthcare for the amplification of whole cells using the GenomiPhi V2 DNA Amplification Kit [175]. However modifications, as described in the methods section, were made to the recommended procedure to keep the volume as small as possible without impacting the efficacy of the procedure. After extraction the entire sample was used for WGA with the AT Kit after which all reactions were quantified with qPCR. As shown in Table 5.5, quantification results were only obtained for two of the 50-cell samples, one 10-cell sample and one 5-cell sample. However, the result for the AT amplified 5-cell sample was only slightly higher than the original starting template amount. All other reactions showed no amplifiable product.

STR analysis of all alkaline lysis AT reactions showed limited success. Two of the 50-cell AT amplified samples had relatively high quantification results of 18ng and 275ng. However, only partial STR profiles were obtained from both samples, with 52% and 61% of the correct alleles recovered respectively. The third 50-cell sample showed a 0ng quantification result, but 20% of the correct alleles were recovered in the STR profile. Only one 10-cell sample showed amplifiable DNA in the qPCR, with a quantification result of 18ng. STR analysis of this sample also gave a partial DNA profile, with 43% of the correct alleles recovered. The other two 10-cell samples had 0ng qPCR results. However, the STR profile from one of these samples showed 13% of the correct alleles. STR analyses of all 5- and 2- and 1-cell AT amplified samples were unsuccessful.

Table 5.5 Total DNA concentrations of LMD cell samples before and after WGA

Number of cells		Before WGA (ng)	One-Tube Extraction & AT-WGA (ng)	Alkaline Lysis & AT-WGA (ng)	Heat Denaturation & AT-WGA (ng)	Spin Column & AT-WGA (ng)
50	1	0.300	1.210	18.00	-	0.000
	2	0.300	0.518	274.5	-	1468.0
	3	0.300	0.500	0.000	-	0.000
10	1	0.060	0.016	18.00	0.000	-
	2	0.060	0.043	0.000	0.000	-
	3	0.060	0.139	0.000	0.000	-
5	1	0.030	0.036	0.000	0.000	-
	2	0.030	0.026	0.040	0.000	-
	3	0.030	0.000	0.000	0.000	-
2	1	0.012	0.049	0.000	0.000	-
	2	0.012	0.070	0.000	0.000	-
	3	0.012	0.000	0.000	0.000	-
1	1	0.006	0.000	0.000	0.000	-
	2	0.006	0.028	0.000	0.000	-
	3	0.006	0.011	0.000	0.000	-

- Method was not performed for this sample

There are several reasons that could explain the limited quality STR results achieved by LMD samples that have undergone WGA. Improper dilution of the WGA product could result in too much template in the reaction resulting in amplification failure. However, all dilutions for STR analysis were based on real time quantification results. The qPCR was performed on 1:100 dilutions of the WGA products, which should have been sufficient to allow for amplification of the WGA product. However, almost all samples gave total yields of less than 100pg. Therefore 1µl of neat product was used for STR analysis.

The high level of salt in the sample after the alkaline lysis procedure or extraction chemicals used in the One-Tube method may be inhibiting the WGA from performing optimally. Alternatively, the alkaline lysis method may not be completely efficient in disrupting the cells and denaturing the DNA for WGA. Another explanation could be that there is an element of the cell samples that is inhibiting the WGA reaction, independent of the extraction method used. It seems unlikely that the 1x TE buffer the cells were collected in would be the cause of inhibition since this did not negatively affect the STR reaction and is the recommended buffer for genomic DNA dilutions that are to be amplified with traditional WGA procedures [175]. However, cells collected using other LMD methods that do not require a high volume of liquid to be in the cap for cell collection have demonstrated successful WGA [94-96]. This indicates that the high TE concentration in the collection buffer could be playing an inhibitory role.

A component of the cells themselves may also be inhibiting the AT reaction. Typical DNA extraction methods involve disrupting the cell membrane and removing all cellular components apart from the DNA, which is generally eluted into TE buffer or water. Since the One-Tube and the alkaline lysis methods retain the sample in the same tube for collection, extraction and WGA, any inhibitory component that would normally be removed by the extraction procedure would be still present in the sample. The inhibitor levels would likely be higher in 50-cell samples, which could explain why poor results are achieved despite the high starting template amount. In 1- or 2-cell samples, inhibitor levels may be lower, but STR profiling would be limited by the low starting template.

To determine if any of the chemicals or salts used in the One-Tube or alkaline lysis methods were inhibiting the AT reaction, three of each 1-, 2-, 5-, 10-cell samples were also extracted using a simple heat denaturation method. However, real time PCR quantification of AT amplified samples after heat denaturation showed that none of the reactions contained amplifiable DNA. As expected, STR analysis of all AT amplified samples was also unsuccessful. To further assess the effect of the high salt concentration from the alkaline lysis procedure on the AT reaction, one 10ng genomic DNA sample in 1x TE buffer was subjected to the alkaline lysis method prior to AT WGA. Real time PCR quantification of the AT product showed a total concentration of 1.868µg of DNA. A 500pg dilution of this product was used for STR analysis and the resulting profile showed 100% allele recovery. This indicates that it is unlikely that the salt concentration from the alkaline lysis procedure is limiting the efficiency of the AT reaction of the LMD cells.

The efficiency of the alkaline lysis method in disrupting the cells was also investigated. One 10-cell sample was extracted using the alkaline lysis procedure and this extract used for STR analysis only. The resulting STR profile showed 65% of the correct alleles recovered, which is lower than the average allele recovery of 80% seen in the 10-cell samples extracted using the One-Tube method that were directly amplified with the 30-cycle PowerPlex ESI 16 reaction. This indicates that the alkaline lysis procedure may not be as efficient as the One-Tube method for extracting DNA from cells for STR analysis. However, this result was higher than the allele recovery seen in any of the AT amplified samples, indicating that there should be sufficient DNA released from the alkaline lysis procedure to be used as template for the WGA reaction.

To assess whether a product of the AT reaction was inhibiting the STR reaction 2µl of the 50-cell AT amplified sample that gave a 0ng quantification was mixed with 400pg of genomic DNA and this mixture was used as the template for STR analysis. Results showed 100% of the genomic DNA alleles recovered, with a mean peak height of 2467 RFU, indicating that the AT reaction does not contain inhibitors for the STR reaction.

5.3.6 Whole genome amplification with QIAamp® DNA Micro spin column extraction

Three 50-cell samples were also extracted using the QIAamp® DNA Micro spin columns. Two of the extracted samples were used directly for STR analysis. However, this was unsuccessful, with both profiles showing no alleles recovered. This indicates that much of the DNA from limited cell samples may be lost in this extraction procedure. The remaining column extracted sample was divided into three aliquots and each amplified first with the AT Kit. The sample was divided to accommodate the maximum volume that can be added to each AT reaction. Each of the three aliquots were quantified after WGA followed by STR analysis. Since this method should remove any inhibitors from the final extract, with DNA eluted into sterile water, AT amplification may have more success compared to the single tube extraction methods. However, real time PCR quantification showed the AT amplification achieved limited success, with only one of the three aliquots showing a result, with a concentration of 1.5µg (Table 5.5). This sample was then diluted to 500pg for STR analysis. The remaining two aliquots showed concentrations of 0ng, and therefore 1µl of the neat product was used as template for STR analysis.

Each of the extracts produced partial STR profiles. The sample with the high quantification result showed the lowest percentage of alleles recovered at 26%, which is surprising considering the reaction should contain 500pg starting template. The other AT samples showed 52% and 43% of the correct alleles recovered. However, numerous artefacts such as over amplified alleles, high stutter and pull up were observed in all profiles in conjunction with stochastic variation such as allele and locus drop out and peak imbalance. These results indicate that the AT reaction is preferentially amplifying regions of the DNA, likely due to the low starting template from dividing the sample, and the qPCR is therefore not accurately quantifying the amplified product. As such, this column extraction method is not ideal for LMD samples, particularly when the cell numbers are limited.

5.4 Conclusion

Overall, these results show that STR analysis of low numbers of LMD cells is, and will likely continue to be, improved by the use of recently developed high sensitivity STR chemistries combined with single tube extraction methodologies. This study shows that complete STR profiles can be obtained from as little as 10 cells using the manufacturer's recommended protocol or 5 cells when amplified with an increased cycle PCR. Increasing the number of cycles did not appear to significantly affect the allele balance in the profiles, with similar PHR averages obtained for the standard and increased cycle profiles. WGA of the cell samples was largely unsuccessful in this study. This is likely due to an inhibitory component in the cells or an inability of the examined extraction methods to disrupt the cells and denature the DNA sufficiently for use with the AT Kit. Further work would need to focus on improving the compatibility of LMD and WGA, as both techniques used together would be a great benefit for situations when the source material is limited.

CHAPTER 6

FORENSIC MITOCHONDRIAL DNA ANALYSIS OF LOW TEMPLATE DNA

6.1 Introduction

Mitochondrial DNA (mtDNA) typing is used routinely in forensic analysis to help identify biological samples when the nuclear DNA template amount is too low or too degraded for conventional STR analysis. Sample types suitable for mtDNA analysis can include bone, hair, blood, saliva, teeth, fingernails and even faeces [132, 137-149]. While there are only two copies of the genomic DNA per cell, depending on the type of cell examined, there are hundreds to thousands of copies of the mitochondrial genome per cell resulting in increased sensitivity of mtDNA assays [135]. The circular structure of mtDNA may also protect it from degradation, further increasing the likelihood of obtaining a result when autosomal STR typing is problematic [136].

Traditionally, the mechanism for mtDNA analysis is DNA sequencing of the hypervariable regions, HV1 and HV2, where all polymorphisms in the fragment can be detected [132]. Due to the maternal mode of inheritance of mitochondrial DNA [152], and the fact that common mtDNA haplotypes can be found in various populations [156], mtDNA sequencing may not yield the magnitude of certainty required for positive identification providing a lower power of discrimination. Furthermore, mtDNA sequencing does not allow the resolution of mixture samples and can be complicated by the presence of heteroplasmy [157]. This is particularly disadvantageous for LTDNA samples as many contain DNA from more than one individual. However, this could be improved if single cells could be examined. Collection of individual cells using techniques such as LMD would give confidence that the haplotype obtained came from a single individual, provided no contamination had occurred.

Methods to improve the mtDNA yield prior to sequencing could also be useful for the analysis of low template samples. WGA using MDA has been applied to blood and tissue samples for detection of cancer causing mtDNA mutations in whole genome sequencing [176]. This procedure showed that WGA could be successful, displaying concordance between mtDNA samples sequenced with and without prior WGA, when up to 20ng of genomic DNA was used [176]. However, this amount of starting material is clearly not available in low template samples. WGA has also been applied to

artificially degraded samples prior to HV1/HV2 sequencing with limited success [110]. WGA methods tended to reduce the amplicon size of the degraded DNA fragments making them unsuitable for sequencing [110]. However, while low template and degraded DNA often produce similar results, LTDNA can still contain intact genetic material. Therefore WGA procedures may have more success when applied to LTDNA prior to sequencing.

Whole mtDNA genome sequencing has also been suggested as a method of increasing the discriminating power of mtDNA [156, 164-167]. Recently developed massively parallel sequencing (MPS) technologies have the potential to provide faster and less expensive sequencing compared to traditional sequencing methods [165-167]. Furthermore, MPS technologies can achieve greater coverage in single reactions [165-167]. The data produced by MPS has shown a high level of consistency with Sanger-type sequencing methods [167]. However, many of the discrepancies observed were related to the MPS analysis software's alignment algorithms, indicating room for improvement in this area [167].

This chapter aimed to examine the applicability of mtDNA sequencing for LTDNA samples. HV1 and HV2 regions were sequenced using cycle sequencing. Serial dilutions of genomic DNA were examined to determine the limit of detection of the sequencing procedure. WGA techniques were also be investigated, with kits targeting both nuclear DNA and mtDNA as well a kit that specifically amplifies mtDNA applied to low template samples. Mitochondrial sequencing was also performed on samples containing various numbers of LMD cells to determine the compatibility of LMD and mtDNA sequencing. WGA that specifically targets the mtDNA was also applied to LMD cells prior to sequencing.

6.2 Methods

6.2.1. Genomic DNA sample preparation

This project was approved by the Bond University Human Research Ethics Committee (BUHREC), approval number RO743. Buccal swabs were provided by 10 anonymous donors with informed consent. DNA was extracted using the BioRobot EZ1® Workstation with the EZ1® DNA Tissue Kits (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. DNA extracts were quantified in triplicate using the method described in section 2.2.1. Based on the quantification results, DNA from all individuals was diluted serially to 10ng/µl, 1ng/µl, 100pg/µl, 10pg/µl, 1pg/µl, 0.1pg/µl, 0.05pg/µl and 0.01pg/µl.

6.2.2 Laser microdissection

Sample preparation, slide preparation and LMD were carried out according to the methodologies described in Chapter 5, with four of each 1, 2, 5 and 10 buccal cells groups collected. Cells were extracted using the modified alkaline lysis procedure also described in Chapter 5.

6.2.3 Whole genome amplification

Low template DNA samples of 10pg/µl were amplified in duplicate using the GenomiPhi V2 DNA Amplification Kit according to manufacturer's instructions and the novel AT Kit as described in Chapter 4 (GE Healthcare, Buckinghamshire, England). Reactions were performed using a GeneAmp® PCR System 9700 (Life Technologies, Carlsbad, CA, USA). After WGA, samples were diluted 1:100 for mtDNA sequence analysis.

Low template DNA samples of 60pg/µl, 30pg/µl, 12pg/µl, 6pg/µl, 1pg/µl, 0.1pg/µl, 0.05pg/µl and 0.01pg/µl were amplified in duplicate using the REPLI-g Mitochondrial DNA kit (QIAGEN) according to manufacturer's instructions. Samples with 60pg/µl

down to 6pg/µl starting template were diluted 1:1000 for mtDNA sequencing, while samples with 1pg/µl down to 0.01pg/µl were diluted 1:100 for mtDNA sequencing. Two of each 1-, 2-, 5- and 10-cell LMD samples were also amplified using the REPLI-g Mitochondrial DNA kit using the recommended protocol. One of each 1-, 2-, 5- and 10-cell amplified sample was diluted 1:1000 for mtDNA sequencing while the remaining samples were diluted 1:100.

6.2.4 Mitochondrial DNA sequencing

Mitochondrial DNA hypervariable regions were sequenced using the BigDye® Direct Cycle Sequencing Kit (Life Technologies) according to manufacturer's instructions. Initially, the HV1 and HV2 regions were amplified in four individuals using the recommended template amount of 4ng. To determine the lower limit of the kit, DNA from one of the individuals was also diluted to 1ng/µl, 100pg/µl, 10pg/µl, 1pg/µl, 0.1pg/µl, 0.05pg/µl and 0.01pg/µl and 1µl of each dilution used for HV1 cycle sequencing. HV1 sequences were then obtained for 1pg/µl, 0.1pg/µl, 0.05pg/µl and 0.01pg/µl dilutions from nine individuals. HV2 sequencing was also performed on all ten individuals using 0.1pg of starting template.

HV1 sequencing was performed on two of each 1-, 2-, 5- and 10-cell LMD samples and one DNA dilution with the equivalent amount of genetic material (6pg, 12pg, 30pg and 60pg respectively). HV1 sequences were also generated for samples that underwent WGA as described in section *6.2.3*.

All samples underwent an initial PCR using M13 tailed primers targeting either the HV1 or HV2 region. Primers for HV1 and HV2 were obtained from published data [144] and are shown in Table 6.1. Cycle sequencing was then performed with the M13 forward or reverse primer. Purification of the mtDNA amplicons was performed with the BigDye XTerminator® Purification Kit (Life Technologies) according to the manufacturer's protocol.

Table 6.1 Primer sequences for mitochondrial DNA hypervariable regions

Region	Primer Sequence*	Fragment Size
HV1	**F-15971 :**	
	5'-TGTAAAACGACGGCCAGTTTAACTCCACCATTAGCACC-3'	439bp
	R-16410 :	
	5'-CAGGAAACAGCTATGACCGAGGATGGTGGTCAAGGGAC-3'	
HV2	**F-15 :**	
	5'-TGTAAAACGACGGCCAGTCACCCTATTAACCACTCACG-3'	374bp
	R-389 :	
	5'-CAGGAAACAGCTATGACCCTGGTTAGGCTGGTGTTAGG-3'	

* M13 sequencing tails indicated in red

Sequences for all samples were obtained using the 3130 Genetic Analyser (Life Technologies). Samples were injected and analysed using the recommended 'RapidSeq36' instrument protocol and 'BigDyeDirect' analysis protocol provided by Life Technologies. Sequences were aligned using Bio Edit software [177] and the Revised Cambridge Reference Sequence (rCRS) [133, 134]. Sequencing results were classified in one of four ways. The first classification was "Over-amplified sequence", which describes sequences that contain numerous instances of dye bleed through due to the abundance of starting template. The second classification was "Complete, good quality sequence". These samples showed the entire HV1 or HV2 sequence without any CE artefacts. The third category was "Sub-optimal sequence" which included samples that showed all or the majority of the sequence at a low level but also contained some CE artefacts such as dye blobs. The final classification was "Unsuccessful amplification" for sequences that showed less than 100 base pairs or no sequence. Examples of the first three categories can be seen in Figure 6.1.

6.3 Results and Discussion

6.3.1 Sensitivity testing

HV1 and HV2 sequencing was initially performed on DNA from four individuals using the BigDye® Direct Cycle Sequencing Kit recommended starting template of 4ng genomic DNA, which is the amount of genetic material that would be found in approximately 667 diploid cells. Assuming that each cell has approximately 1000 mitochondria, 4ng genomic DNA could contain up to 667 000 copies of the mitochondrial genome, an overly excessive amount for PCR and CE detection techniques. It was not surprising then that results showed sequence profiles with over amplified peaks and extremely high pull up, confirming that this starting template amount was too high. The sensitivity of the BigDye® Direct system is also likely quite high as the sample stays in the same tube through PCR, cycle sequencing, amplicon purification and CE, negating any loss that normally occurs through pipetting or changing tubes.

To determine the lower DNA limit of the BigDye® Direct system, DNA from one individual was diluted to 1ng/µl, 100pg/µl, 10pg/µl, 1pg/µl, 0.1pg/µl, 0.05pg/µl and 0.01pg/µl, with 1µl of each dilution used for HV1 cycle sequencing. The HV1 region was chosen as this is the larger of the two regions and would best reflect the capabilities of the kit. Approximate mtDNA genome copies for each genomic DNA dilution can be seen in Table 6.2.

Sequencing results for the 1ng, 100pg, 10pg and 1pg samples showed over amplified profiles. This is not surprising since, as can be seen in Table 6.2, 1pg genomic DNA could still contain around 167 copies of the mtDNA genome. Results from the three samples with the lowest template amounts all showed complete HV1 profiles. However, the 0.05pg sample profile was of sub-optimal quality, with small sequence peaks and three large CE artefacts seen in the electropherogram, one of which can be seen in the third panel of Figure 6.1.

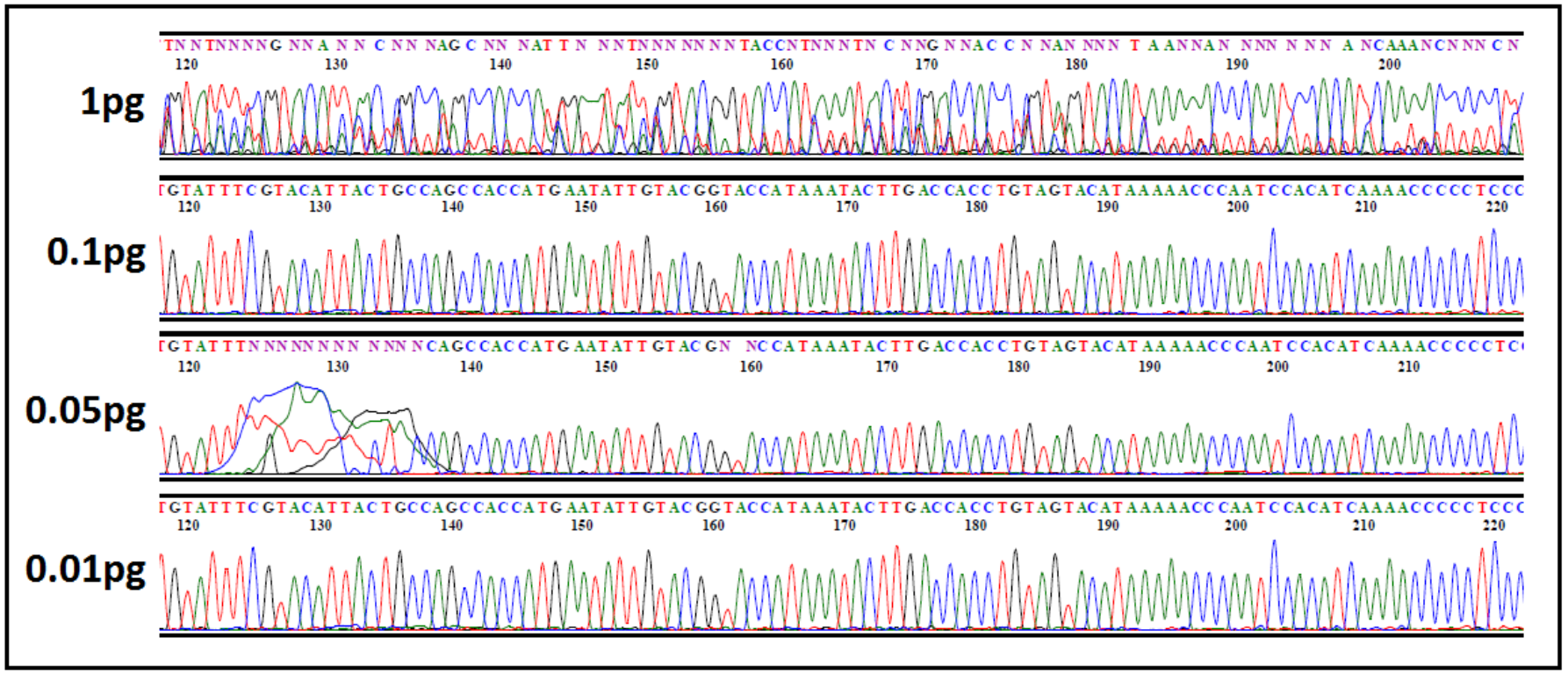

Figure 6.1 HV1 sequences from DNA dilutions. HV1 sequencing using 1pg genomic DNA starting template shows an over amplified sequence with CE artefacts present. Sequencing using 0.1pg and 0.01pg genomic DNA starting templates shows complete, good quality sequence. Sequencing using 0.05pg starting template shows a sub-optimal quality sequence, with small peaks and CE atrefacts present.

Table 6.2 Mitochondrial DNA copies in genomic DNA dilutions

Genomic DNA amount	Number of diploid cells / genomic DNA amount	Approximate mtDNA copies*
4ng	667	667000
1ng	167	166700
100pg	16.7	16670
10pg	1.67	1667
1pg	0.167	166.7
0.1pg	0.0167	16.67
0.05pg	0.0083	8.3
0.01pg	0.00167	1.667

* Assumes 1000 copies of the mitochondrial genome per cell

The amount of mtDNA per cell can vary from person to person, therefore the HV1 region was sequenced for nine other individuals using 1pg, 0.1pg, 0.05pg and 0.01pg. Results for all ten individuals can be seen in Table 6.3. Overall, six of the ten 1pg samples resulted in over-amplified sequence profiles while the remaining four showed good quality HV1 sequences. When the template was reduced to 0.1pg, nine samples showed complete HV1 sequences, although one of the profiles was of sub-optimal quality. One 0.1pg sample was unsuccessful. With 0.05pg starting template, 5 of the 10 samples gave complete, good quality HV1 sequences, four were sub-optimal quality profiles and one was unsuccessful. Using the lowest template amount - 0.01pg - three samples gave complete, good quality sequences, three gave sub-optimal quality sequences and four were unsuccessful. Such results are an improvement compared to a previous mtDNA sensitivity study, which demonstrated HV1 sequencing with 0.03pg genomic DNA using a nested PCR amplification strategy [143].

Since the majority of samples gave complete HV1 sequences with 0.1pg, this dilution was used for sequencing of the HV2 region. As seen in Table 6.3, all samples produced complete, good quality HV2 profiles. Comparisons of the HV1 and HV2 sequences to the rCRS are shown in Table 6.4.

The ability to gain complete control region sequences from such small amount of DNA means that very little of the sample is consumed by mtDNA sequencing. This is promising for LTDNA samples, as the remainder could be used to attempt STR analysis or for the determination of the often critical question of tissue of origin. Determining the tissue source of forensic evidence samples can be great importance in crime reconstruction. Tissue determination techniques, such as mRNA [66, 67] and microRNA analysis [68] or DNA methylation studies [69] are being currently being investigated, and could be applied to LTDNA samples. Such methods have high specificity, fast analysis times and do not consume large portions of the sample. Also, since these methods use PCR based techniques they could be integrated easily into the current forensic DNA workflow [17].

Table 6.3 Sequence quality of LTDNA dilutions

Sample	**HV1**				**HV2**
number	1pg	0.1pg	0.05pg	0.01pg	0.1pg
1	xs	++	+	++	++
2	xs	++	++	++	++
3	++	++	+	++	++
4	++	+	+	+	++
5	xs	++	++	+	++
6	xs	++	++	-	++
7	xs	++	++	+	++
8	++	-	+	-	++
9	++	++	++	-	++
10	xs	++	-	-	++

xs Over amplified sequence, ++ Complete, good quality sequence, + Sub-optimal quality sequence, - Unsuccessful amplification.

Table 6.4 HV1 and HV2 sample comparisons to the Revised Cambridge Reference Sequence (rCRS)

	16129	16158	16162	16172	16183	16189	16193	16209	16223	16255	16263	16270	16293	16311	16320	73	150	152	153	185	195	198	199	203	204	225	226	250	263	309.1	315.1
rCRS	**G**	**A**	**A**	**T**	**A**	**T**	**I**	**T**	**C**	**G**	**T**	**C**	**A**	**T**	**C**	**A**	**C**	**T**	**A**	**G**	**T**	**C**	**T**	**G**	**T**	**G**	**T**	**T**	**A**	**I**	**I**
1	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	C
2	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	G		C
3	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	G		C
4	.	.	.	.	.	.		.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G		C
5	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	G		C
6	A	.	.	C	.	.		.	T	.	.	.	.	C	.	G	.	.	.	.	.	.	C	A	C	.	.	C	G		C
7	.	G	.	.	.	.		.	T	.	.	.	.	.	T	G	T	.	.	.	C	T	.	.	.	.	.	.	G		C
8	.	.	G	.	.	.		C	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	G		C
9	.	.	.	.	.	.		.	.	.	.	T	G	.	.	G	T	.	.	A	.	.	.	.	.	.	.	.	G	C	C
10	.	.	.	.	C	C	C	.	.	A	.	.	.	.	.	G	.	.	G	.	C	.	.	.	.	A	C	.	G		C

A dot (.) indicates no difference to the rCRS. Base calls indicate a substitution at that position. An insertion compared to the rCRS is designated as the appropriate position number followed by a dot, and indicated by an (I) in the rCRS.

6.3.2 Mitochondrial DNA sequencing of LMD cells

HV1 sequencing was also performed on groups of LMD cells. After alkaline lysis extraction, 1µl of each cell sample (1/10 of the total sample volume) was used for the sequencing reaction. Results for two of each 1-, 2-, 5- and 10-cell samples can be seen in Table 6.5. Apart from the 5-cell samples, which both produced good quality sequence, only one of each cell number produced the complete sequence, with the 1-cell sample sequence of sub-optimal quality. This is surprising considering previous results showed complete mtDNA sequences could be obtained from significantly lower DNA amounts (seen in Table 6.3). 1µl of the 10-, 5- 2- and 1-cell samples should contain approximately 1000, 500, 100 and 50 copies of the mtDNA genome respectively, which previous results showed is more than what is necessary for sequencing and should have resulted in some measure of over amplification. However, the results did not show this over amplification, and were more consistent the results from lower template samples (0.1pg to 0.01pg) seen in Table 6.3.

Table 6.5 Sequence quality of lasermicrodissected cells

Number of Cells	HV1 Trial 1	HV1 Trial 2
10	++	-
5	++	++
2	++	-
1	+	-

++ Complete, good quality sequence, + Sub-optimal quality sequence, - Unsuccessful amplification.

One reason for these poor results could be that the alkaline lysis extraction method is not sufficient to disrupt the cells. However, this is unlikely considering some of the samples did display results. Furthermore work in the previous chapter (see Section 5.3.4) showed that this extraction method was compatible with PCR analysis. Additionally, more than 1µl of the cell sample may be necessary to obtain quality data. However, as mentioned above, 1µl should contain sufficient mtDNA copies for analysis. Another possible explanation is that the samples may contain an inhibitor that remains in the sample through the extraction process, since the entire reaction is

performed in a single tube. As a comparison sequencing was performed on cell-equivalent DNA amounts. Samples containing a total DNA concentration of 60pg, 30pg, 12pg and 6pg in 10µl volumes were generated and 1µl of each used for cycle sequencing. As expected, complete HV1 sequences were generated from each of the samples, with the 60pg, 30pg and 12pg samples producing over amplified results demonstrating too much DNA present. This indicates that whole cell samples were not performing optimally.

The ability to sequence single cells would be of great benefit to forensic analysis since one of the key issues with mtDNA typing is the inability to resolve mixture samples. Therefore future work should be done to investigate different extraction methodologies so that LMD can be compatible with sequencing chemistries.

6.3.3 Whole genome amplification of DNA dilutions

This study also aimed to examine the ability of WGA techniques to amplify low template mitochondrial DNA for sequencing. Initially, 10pg samples were amplified in duplicate with two WGA kits, the GenomiPhi V2 DNA Amplification Kit and the AT Kit, which target nuclear and mitochondrial DNA. WGA reactions were diluted 1:100 and 1µl used for HV1 cycle sequencing. As shown in Table 6.6, all 10pg samples from both kits produced complete profiles of high quality.

Table 6.6 Sequence quality of LTDNA after whole genome amplification

Sample		**HV1 Sequence Quality**
10pg with GenomiPhi:	1:	++
	2:	++
10pg with AT:	1:	++
	2:	++

++ Complete, good quality sequence

The REPLI-g Mitochondrial DNA kit, which is a mitochondrial specific WGA (mtWGA) kit, was also examined. With this kit, low template DNA samples containing 60pg/µl, 30pg/µl, 12pg/µl, 6pg/µl, 1pg/µl, 0.1pg/µl, 0.05pg/µl and 0.01pg/µl were amplified in duplicate, with 1µl of each used for HV1 analysis. Amplified samples from 60pg to 6pg of starting template were diluted 1:1000 as per manufacturer's instructions for cycle sequencing while mtWGA samples from 1pg to 0.01pg starting template were diluted 1:100. As shown in Table 6.7, both 60pg, 30pg and 12pg samples produced over amplified sequences, indicating that a higher dilution factor was required. When only 6pg was used as starting template for mtWGA, one reaction produced a good quality profile while the other produced a sub-optimal quality profile, indicating that, while the mtWGA had been successful, at this level samples should not be too diluted prior to sequencing. Only one of the 1pg samples produced a complete sequence, and all 0.1pg, 0.05pg and 0.01pg samples were unsuccessful. This suggests that the mtWGA reaction was not successful, rather than the 1:100 dilution being too high, since mtDNA sequences could be obtained from these samples without WGA. If the WGA reaction was successful there should have been sufficient product in the dilution for sequencing. Overall, these results are promising, in that high quantities of amplifiable mtDNA template are being generated from as little as 1pg of genomic DNA with the REPLI-g Mitochondrial DNA kit. This is especially notable considering the recommended genomic DNA input for the mtWGA kit is 10ng.

6.3.4 Whole genome amplification of LMD cells

The mtWGA kit was also used with two of each 1-, 2- 5- and 10-cell samples. However, as shown in Table 6.7, all but one 10-cell sample were unsuccessful. The results are not surprising considering WGA of LMD cells was not previously successful (See Chapter 5, Section 5.3.4). As suggested in Section 5.3.4 the failure of the WGA could be due to poor extraction or inhibitors not being removed through the extraction and subsequent dilution steps that genomic DNA was subjected to.

Table 6.7 Sequence quality of LTDNA and laser microdissected cells after mitochondrial whole genome amplification

Sample	Trial 1	Trial 2
60pg	xs	xs
30pg	xs	xs
12pg	++	xs
6pg	+	++
1pg	++	-
0.1pg	-	-
0.05pg	-	-
0.01pg	-	-
10 Cells	xs	-
5 Cells	-	-
2 Cells	-	-
1 Cell	-	-

xs Over amplified sequence, ++ Complete, good quality sequence, + Sub-optimal quality sequence, - Unsuccessful amplification.

The ability to produce large quantities of mtDNA template would be of great benefit in forensic analysis, particularly in relation to emerging sequencing technologies. Massively parallel sequencing platforms are now able to sequence the entire mitochondrial genome in a single reaction [165-167]. Since another of the key challenges associated with mtDNA sequencing is the relatively low power of discrimination compared to traditional STR analysis, the ability to sequence the entire genome would give more information for forensic identification purposes. However, such technologies require a starting template of 1-5ng genomic DNA for an initial long-range PCR, with 100ng of the PCR product needed for the sequencing reaction [178]. Low template DNA samples would therefore not be ideal for these purposes unless a technique such as WGA could be used to amplify the template prior to sequencing. Results presented here suggest that either traditional WGA kits or mitochondrial specific WGA kits could be used for such purposes.

6.4 Conclusion

In conclusion these results have confirmed that low template DNA samples containing as little 0.1pg to 0.01pg genomic DNA can be successfully used for mitochondrial DNA sequencing. The ability to perform mtDNA sequencing with minimal sample consumption could allow for additional testing to occur, such as tissue of origin determination, which could be of great importance for crime reconstruction.

In this study, limited success was achieved when sequencing LMD cells. However this was likely due to the incompatibility of the alkaline lysis extraction technique with the sequencing chemistry. Future work should continue to explore other cell extraction methodologies for downstream mtDNA sequencing. The ability to amplify single cells would be of great benefit since it would be known that the resulting sequence originated from a single contributor.

This study also showed that WGA techniques could be applied to low template samples for successful mtDNA sequencing. Samples containing as little as 10pg genomic DNA were successfully amplified with WGA kits that target both nuclear and mtDNA. After WGA, 1µl of a 1:100 dilution of the reaction contained sufficient mtDNA for HV1 sequencing. Samples containing only 0.1pg genomic DNA amplified with a mitochondrial specific WGA kit also produced sufficient template in a 1:100 dilution for successful HV1 sequencing. Such results show promise for massively parallel sequencing technologies that can amplify the entire mtDNA genome in a single reaction but require higher starting template amounts. The application of mitochondrial WGA to LMD cells was generally unsuccessful in this study and should be another focus of future work.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

7.1 Introduction

Issues associated with LTDNA analysis have been well documented since the technique was initially proposed in the mid-1990s. However, until the Omagh Bombing trial in 2007 there was limited challenge to the use of LCN for legal purposes. Concerns raised during this trial, and the subsequent brief suspension of the technique in the UK, brought LTDNA analysis into the spotlight. This project aimed to examine the current methods for LTDNA analysis and investigate strategies for improving the results that could be achieved with limited starting template.

The most common method for genotyping LTDNA is the combined LCN and replicate analysis approach. Despite its widespread use, little empirical evidence has been provided to confirm that this method does indeed provide the most informative DNA profiles. Therefore, initial studies involved a direct comparison between consensus profiles derived from replicates of divided DNA samples with profiles obtained using the entire low template extract to determine which method gave the most information from the limited samples.

Methods to increase the DNA yield prior to traditional STR analysis were also investigated. Initial work examined a non-exponential Pre-PCR amplification that produced additional allele copies in each low template sample before undertaking traditional DNA typing methods. Various WGA kits were also examined to assess their ability to copy the entire LTDNA sample in a representative fashion. Modifications to the recommended WGA protocols were investigated to determine if allele recovery and balance could be improved.

LMD was used to isolate single and small numbers of cells for forensic analysis. Various extraction and amplification methodologies were examined to determine optimal techniques for use with LMD cells. STR analysis was performed using both standard and LCN PCR techniques. Cell samples were also subjected to WGA prior to

STR analysis to determine if any could improvements could be made to the allele recovery and balance in the final profile.

As an alternative to STR analysis, mtDNA control region sequencing was investigated as a method for LTDNA analysis. Initial sensitivity testing was performed to determine the lowest template amount needed to still produce a complete mtDNA HV1 and HV2 sequence. Techniques previously investigated in this project including WGA and LMD were then combined for use with mtDNA sequencing. DNA dilutions were amplified with various WGA kits prior to sequencing. LMD cells, with and without WGA, were also used as template for the sequencing reaction.

7.2 Analysis of current LTDNA profiling techniques

Low template DNA samples containing 100pg or 25pg genomic DNA were amplified using the PowerPlex® ESI 16 kit with 30 or 34 PCR cycles. 100pg and 25pg DNA samples were then divided into three aliquots for a 34-cycle PCR. A consensus profile was derived that included alleles that appeared in at least two of the replicates. Profiles from the non-split samples were compared to the consensus profiles focusing on peak heights, allele drop out, locus drop out and allele drop in to determine which technique provided the most informative STR profiles.

Results from this study demonstrated that performing standard 30-cycle STR typing on non-split DNA extracts produced profiles with a higher percentage of total loci compared with the consensus profiling technique. Increasing the number of PCR cycles further improved the sensitivity of the reaction, with reduced allele and locus dropout seen for both template amounts compared to both standard-cycle single-reaction profiles and consensus profiles. However, profiles from samples containing 100pg genomic DNA – a template amount on the upper limits of what would be considered low template DNA – contained increased levels of additional alleles, either drop in or stutter. This indicates that increasing the number of PCR cycles to this degree may not actually benefit such samples if a single reaction, rather than replicate analysis, is to be

performed. However, since some allele drop out still occurred in profiles from 100pg amplified with the standard cycle number, there may be some advantage to increased cycle amplification, but perhaps fewer than 34 cycles would be preferable.

Allele drop in was eliminated using the consensus profiling method for both starting template amounts. This has important implications for casework, where errors in the profile interpretation could then lead to false inclusion or exclusion of suspects, or false matches if the profile is subjected to a database search. However, since all additional alleles occurred at heterozygous loci that also showed both correct alleles or were well under a typical peak height ratio threshold of 60% to 70%, these alleles would be interpreted with caution before being attributed to the final profile for a single source profile. All other measures of profile quality were improved when the sample was amplified as a whole, with consensus profiling resulting in the least informative profiles due to increased allele or locus drop out. Replicate analysis also produced more 'incorrect' alleles in the individual profiles used to obtain the consensus profile as a result of increased stutter.

Overall these results indicate that a single standard cycle PCR on the entire sample will produce the most complete profiles when at least 100pg of starting template are available for amplification. When only 25pg of template are available, it would be beneficial to amplify the entire extract with an increased cycle PCR in terms of acquiring a profile with the most information possible. However, since allele and locus drop out and allele drop in can still occur when a low template DNA sample is amplified in a single reaction, a robust statistical analysis model that takes the stochastic effects into consideration must be applied to the data. Statistical tools are being developed that may accommodate issues such as drop in and drop out and these have been implemented in some laboratories [56-61]. Applying such tools to the DNA profile that contains the most information should maximise the evidence.

7.3 Improving DNA yield prior to STR analysis

Two studies were performed to determine if additional starting template could be generated to improve results achieved by traditional STR analysis. In the first study a novel Pre-PCR linear amplification was investigated. The second study involved an analysis of commercial and novel WGA kits using recommended and modified protocols.

7.3.1 Linear Pre-PCR amplification of LTDNA

Low template DNA samples of 100pg, 50pg, 25pg, 12.5pg and 6.25pg were divided into two aliquots. One aliquot was used as template for a PCR using only the forward primer for a single locus, or a primer mix containing all forward primers for loci targeted in the PowerPlex® ESI 16 kit. The remaining aliquot was amplified with the reverse primer for the same single locus or a primer mix containing all reverse primers for PowerPlex® ESI 16 loci. This Pre-PCR amplification was performed using either 10 or 20 cycles. Forward and reverse amplification products were then pooled for use in a standard PCR with the single locus primer pair or PowerPlex® ESI 16 kit Primer Mix. The proposition was that the forward or reverse primer reactions would result in a linear amplification, where a single copy of the targeted allele(s) would be produced with each cycle, rather than the exponential amplification of traditional PCR, and as such should not introduce the same degree of stochastic effects. With additional template copies, STR results may show improved allele recovery without the exacerbated stochastic effects commonly seen with LCN DNA analysis.

Overall this research demonstrated that improved STR profiles from samples with low levels of template can be obtained using Pre-PCR amplification prior to a single locus or multiplex PCR. The 20-cycle Pre-PCR generally provided the highest percentage of profiles with both alleles for the single locus reactions, with all 100pg and 50pg profiles displaying the correct genotype. In the multiplex experiments the 10- and 20-cycle Pre-PCR produced STR profiles with all loci correct in the 100pg samples only. Below this template amount, while there was an increase in allele recovery overall with the Pre-

PCR, none of the control, 10- or 20-cycle Pre-PCR samples showed the complete multiplex STR profile.

Despite the increase in allele recovery the peak height ratios for the Pre-PCR amplified samples were not considerably different compared with control samples using the higher starting template amounts (100pg and 50pg in the single locus experiments, and 100pg, 50pg and 25pg in the multiplex experiments). This indicates that the linear amplification of the Pre-PCR increased the number of template copies available for the PCR without introducing substantial amplification bias for these template amounts. Results were more variable in terms of allele recovery and peak height balance with the lower starting template amounts, indicating that, at least when limited to 20 cycles, the Pre-PCR procedure is not sufficient to improve the number of template copies for the PCR amplification for such low level samples.

The multiplex results have the most implications for forensic case work as multiplex STR profiling is the primary method used for human identification. As such improvements would have to be made before any such procedure could be implemented. In these experiments, only half of the first-round Pre-PCR product was used as the template for the second-round PCR. While this still showed improved allele recovery, results may be further improved if the entire first round PCR product could be used. Future work could therefore involve further reduction of the volume of the first-round Pre-PCR or increasing the volume of the second round PCR so that all of the possible template could be used for the second PCR amplification. Further improvements may also be seen with an increase in the number of Pre-PCR cycles. The linear amplification of the Pre-PCR step did not introduce further stochastic variation compared to samples amplified without Pre-PCR processing when 20 Pre-PCR cycles were used. This bodes well for the possibility for the use of a greater number of Pre-PCR cycles for very low levels of template.

7.3.2 Whole genome amplification

The study of WGA techniques was performed as two sets of experiments. In the initial set of experiments LTDNA samples were amplified with the GenomiPhi V2 DNA amplification kit using the standard protocol and modified protocols to determine if any improvements could be made to the efficiency of the reaction. After these initial experiments were complete, a novel WGA chemistry, called the AT kit, was provided for comparison to the GenomiPhi kit and the REPLI-g Mini kit. Modifications to the AT kit protocol were also assessed to determine if improved amplification efficiency could be achieve. Mixture samples were also analysed with the AT kit to determine if this WGA kit could assist in the recovery of low level contributors

Results from the initial set of experiments showed that the greatest allele recovery was achieved when LTDNA samples were amplified using the standard GenomiPhi protocol prior to STR analysis. Of the modified protocols, the Cycling protocol came closest to the standard protocol, with more correct alleles recovered compared to STR profiles from low template samples that did not undergo prior WGA. The Split and Pool and Half Denatured protocols showed significantly lower allele recovery across all examined starting template amounts compared to the standard and Cycling protocols, with the Split and Pool protocol generally showing the lowest efficiency of the methods. Across all low template amounts the Split and Pool protocol consistently showed less alleles recovered compared to samples that did not undergo prior WGA.

WGA reactions in the initial set of experiments were not quantified after amplification. Instead a blanket 1:100 dilution was applied to all WGA samples and 1µl of this dilution was used for STR analysis. The pronounced drop out and allele imbalance in the STR profiles from Split and Pool and Half Denatured reactions indicate that these two methods produced less amplification product compared to the standard and Cycling protocols. Consequently, the high dilution of the amplification product returned the samples to low template levels, resulting in increased stochastic variation in the STR profiles. It was therefore necessary to quantify all WGA samples prior to STR profiling to ensure the optimum amount of starting template is used so that any WGA bias may

be assessed without the additional complication of LTDNA associated stochastic effects.

In the second set of experiments, all WGA samples were quantified prior to STR analysis. Overall, quantification results showed that the novel AT kit produced the lowest amount of WGA product when LTDNA was used as the starting template. It was surprising then that STR analysis of the WGA products showed allele recovery and peak height ratios were highest in the AT amplified samples, followed by the REPLI-g kit then the GenomiPhi kit. This indicates that, despite the low quantification results, the AT kit is amplifying the LTDNA in the most representative fashion. However, all three chemistries allowed for significantly more alleles to be recovered compared to LTDNA samples that did not undergo WGA prior to STR analysis. Despite having the best results of the three kits, drop out still occurred with the AT kit when less than 50pg of DNA was used as the starting template and significant allele imbalance was seen in some loci. Such stochastic variation can make STR profile interpretation difficult, and therefore further improvement is needed before this WGA procedure could be routinely implemented into the forensic DNA analysis workflow for casework.

Modifications to the AT protocol showed varying results. The Cycling protocol samples consistently showed reduced allele recovery and PHRs compared the standard protocol. However, the Split and Pool Protocol showed equal or slightly higher allele recovery and similar PHRs compared to the standard protocol. This result is surprising considering that the Split and Pool protocol showed the worst results of the examined protocols in the first set of experiments. However, this gives weight to the notion the high dilution used in the first set of experiments contributed significantly to the poor STR profiles, rather than the WGA protocol introducing a high level of amplification bias alone. The slight increase in allele recovery seen in the 10pg starting template amplified with the Split and Pool protocol is promising, suggesting that dividing the reaction allowed for the small amount of template in each aliquot to be amplified in a more representative fashion than would be if the reaction was kept whole for amplification. However, as with the standard protocol samples, since stochastic variation was present in the Split and Pool WGA STR profiles, even if it was at reduced

levels, further improvements are necessary before implementation into routine casework.

WGA of mixture samples using the AT kit showed increased alleles recovered when both contributors were at equal low template levels compared to samples without prior WGA. However, when the mixture samples contained unequal contributions from donors, the WGA reaction preferentially amplified the DNA from the major contributor resulting in a reduced number of minor contributor alleles recovered. This indicates that while WGA may be useful for single source samples or low template mixed samples where donors contribute equal amounts of DNA to the sample, it is not the preferred analysis option for mixtures with major and minor contributors.

7.4 Low copy analysis of intact cells

LMD was used to isolate single and small numbers of buccal cells for use as template for forensic STR analysis. Compared to previous studies on isolated cells, these results show that improved results can be achieved by using recently developed higher sensitivity STR chemistries combined with single tube extraction methodologies. This study showed that complete STR profiles can be obtained from as little as 10 cells using the manufacturer's recommended protocol or 5 cells when amplified with an increased cycle PCR. Increasing the number of cycles did not appear to significantly affect the allele balance in the profiles, with similar peak height ratio averages obtained for the standard and increased cycle profiles.

DNA dilutions containing the equivalent template amount to the LMD cell samples were also analysed. This was done to determine if the intact cell samples were amplifying in the same manner as DNA dilutions. Results showed that overall allele recovery was higher in DNA dilutions across all template amounts with 30 and 34 PCR cycles compared to cell samples. Cell samples contain a known number of genome copies, whereas the dilutions contain an estimated genomic DNA amount based on the quantification of a high template sample. This estimation could be higher or lower than

the actual amount contained in the sample depending on the accuracy of the quantification. However, the higher allele recovery in the DNA dilutions is likely not solely the result of inaccurate quantification. Complete amplification failure was seen in 1-, 2- and 5-cell samples amplified with both 30 and 34 PCR cycles, indicating that either the extraction method is not disrupting the cells sufficiently for STR analysis, or a component of the cells themselves or chemicals involved in the extraction could be inhibiting the success of the reaction.

WGA of the cell samples was largely unsuccessful in this study despite the numerous extraction methodologies investigated. This is likely due to an inhibitory component in the cells or an inability of the examined extraction methods to disrupt the cells and denature the DNA sufficiently for use with the AT WGA kit. Further work would need to focus on improving the compatibility of LMD and WGA, with particular focus on developing an efficient DNA extraction methodology. The ability to successfully amplify the entire genome of a single cell in a representative fashion would be a great benefit for forensic purposes, where template amounts can often be limited. Such techniques would also be of benefit to other scientific fields, such as preimplantation genetic diagnosis or oncogenetics, where numerous tests can often be required from minute or precious samples.

7.5 Alternative markers for LTDNA analysis

Human identification by mtDNA analysis has been routinely used for challenging biological samples that would typically fail with traditional STR analysis due to the limited quantity or quality of the genomic DNA. In this study serial dilutions of genomic DNA were examined to determine the limit of detection of the sequencing procedure. WGA techniques were investigated, with kits targeting both nuclear DNA and mtDNA, as well a kit that specifically amplifies mtDNA only to be applied to low template samples prior to sequencing. Mitochondrial sequencing was performed on samples containing various numbers of LMD cells to determine the compatibility of LMD and mtDNA sequencing. WGA that specifically targets the mtDNA was also applied to LMD cells prior to sequencing.

Results from this study demonstrated that low template DNA samples containing as little 0.1pg to 0.01pg of genomic DNA can be successfully used for mitochondrial DNA sequencing. The ability to perform mtDNA sequencing with minimal sample consumption could allow for additional testing to occur, such as tissue of origin determination. This would be of great benefit since determining the tissue source can be of critical importance for crime reconstruction.

Limited success was achieved when sequencing laser microdissected cells. This was likely due to the incompatibility of the extraction technique used with the sequencing chemistry. Future work should continue to explore this avenue. Since one of the key issues with mtDNA sequencing is the inability to resolve mixture samples the ability to amplify single cells would be of great benefit because it would be known that the resulting sequence originated from a single contributor.

This study also showed that WGA techniques could be applied to low template samples for successful mtDNA sequencing. Samples containing as little as 10pg genomic DNA were successfully amplified with WGA kits that target both nuclear and mtDNA. After WGA, 1µl of a 1:100 dilution of the reaction contained sufficient mtDNA for complete HV1 sequencing. Samples containing only 0.1pg genomic DNA amplified with a mitochondrial specific WGA kit also produced sufficient template in a 1:100 dilution for successful HV1 sequencing. The success of WGA for mtDNA is likely due to its structure and inheritance pattern. Mitochondrial DNA is circular, which would be a benefit for MDA since this was originally designed as a Rolling Circle Amplification for use on circularized DNA probes [116]. Furthermore, since mtDNA is inherited as a single haplotype, issues surrounding heterozygote balance are avoided.

Mitochondrial DNA sequencing is often avoided for various reasons. One such reason is the generally lower power of discrimination compared to traditional STR analysis. Statistical analysis to determine the frequency of an mtDNA type in a population is often limited to the “counting method”, where the number of times a particular type has been observed in various databases is presented [150]. The frequency is therefore

limited by the size of the database(s) used for comparison. However, despite the lower power of discrimination for individual identification, this study has shown that more complete information can be gained from sequencing results compared to STR results from the same amount of LTDNA. Therefore, depending on the starting template amount available for analysis and the size of comparison databases, the mtDNA frequency determined by the counting method could potentially be more discriminating than a statistic conferring rarity of a partial STR profile.

The time and cost associated with mtDNA sequencing is also often noted as a reason to avoid the technique. However standard Sanger-type sequencing uses the same equipment as traditional DNA profiling. Furthermore new mtDNA sequencing chemistries such as those used in this study allow for the reactions to be performed in the same plate for PCR, cycle sequencing, purification and electrophoresis, resulting in a processing time not unlike STR analysis. Another issue with mtDNA is the potential for contamination. However, many of the necessary anti-contamination procedures such as dedicated laboratory areas for pre-and post-PCR work and use of single use lab coats, gloves, masks and caps are already in use in many forensic laboratories. Furthermore, if a laboratory is to dedicate areas for LTDNA work, then this space would be equally compatible with mtDNA analysis.

Results presented here also show promise for emerging massively parallel sequencing technologies. Such technologies can amplify the entire mtDNA genome on a single chip but require higher starting template amounts than traditional sequencing. In this study extremely low template DNA was successfully amplified with an mtDNA specific WGA kit, and this amplification product is potentially a viable sample type for emerging sequencing techniques.

7.6 Future directions

Genotyping of low template DNA will likely continue to be a desire of police and forensic service providers. Therefore efforts must be made to ensure that the methods used to analyse LTDNA are reliable and provide the most information possible. It is unlikely that laboratories currently practicing LCN with replicate analysis will discontinue these methods since repetition is used to justify reliability, despite the replicates producing different DNA profile results. However, it is worth considering the loss of information that occurs when an already low template sample is divided for amplification and the additional information that can be gained if a LTDNA sample is amplified in a single reaction. The introduction of statistical software that accommodates stochastic variation into forensic laboratories is a positive development. The use of such tools could be maximised by applying them to the most informative profile that can be achieved from the limited template sample. It is of note that the software interpretation tools analyse a single, not a consensus, profile and the analysis of LTDNA in one reaction therefore seems desirable.

The ability to genotype single or small numbers of whole cells would be of great benefit for forensic investigation. Using LMD to isolate specific cells can, in certain circumstances, eliminate issues with mixture analysis and interpretation. However, the currently available STR chemistries are not able to amplify a single cell so that all loci are represented completely in the DNA profile. Therefore, either a more robust STR chemistry must be developed or a method to first increase the allele copies or the entire genome is required for successful STR typing. The linear Pre-PCR amplification presented in the thesis shows promising results that could be developed for single cell analysis. Efforts should also be made to develop extraction techniques for single or small numbers of intact cell that can accommodate the volume necessary for LMD collection and are compatible with WGA. Emerging WGA chemistries must also be optimised for single cell analysis to eliminate the amplification bias that occurs with most current WGA methods.

Whole mitochondrial genome sequencing using massively parallel sequencing technologies is another promising area for investigation. Amplification of limited template using mtDNA specific WGA could enable LTDNA to become a viable sample type for whole genome sequencing. Sequencing the entire mtDNA genome as opposed to the control region alone would provide significantly more information for human identification, and would therefore make mtDNA a sound alternative to traditional autosomal STR analysis.

7.7 Final conclusions

The overall aim of this research was to examine the current methods of LTDNA analysis and investigate methods which could produce improved results for human identification from samples with a limited starting template. A comparison of consensus profiles to profiles obtained using the entire LTDNA samples in a single reaction was performed. Methods to increase the DNA yield prior to STR analysis were investigated. LMD was examined as a potential collection method for single and small numbers of cells to be used as template for STR analysis. Mitochondrial sequencing was also investigated as an alternative to traditional autosomal STR profiling for human identification from LTDNA.

The specific outcomes of this thesis were:

1. Direct comparison of consensus STR profiles with profiles from non-split samples demonstrated that a considerable loss of information occurred when LTDNA was divided for amplification
2. Applying a linear Pre-PCR amplification to low template samples improved STR profile results in both single locus and multiplex reactions.
3. A novel WGA kit provided by GE Healthcare was shown to amplify LTDNA in a manner that produced superior STR results compared to currently available commercial kits.
4. Real time PCR quantification of WGA products was shown to be problematic. Using a qPCR primer for a region located close to the centromere provided an

under-estimated quantification value, resulting in excess template being added to the STR reaction.

5. Splitting a WGA reaction prior to amplification then pooling the aliquots for STR analysis showed slightly improved profiles compared to a single amplification of the entire reaction.
6. WGA with the novel AT kit allowed for increased allele recovery in low template two-person mixture samples when both parties provided equal contributions to the mixture. WGA of samples where parties have provided major and minor contributions to the mixture resulted in preferential amplification of the major contributor.
7. LMD was successfully performed to isolate single and small numbers of buccal cells.
8. Extraction of LMD cells using the One Tube method followed by amplification with the PowerPlex® ESI 16 STR kit using standard and LCN cycling conditions showed some success. Complete STR profiles were obtained with 10 cells using standard cycling conditions or 5 cells using LCN cycling.
9. None of the examined extraction methodologies used for cellular disruption of LMD samples (the One Tube method, alkaline lysis, heat denaturation and spin column extraction) were compatible with whole genome amplification using the AT kit.
10. Mitochondrial control region sequencing was performed using as little as 0.01pg to 0.1pg genomic DNA.
11. WGA of 10pg genomic DNA using the GenomiPhi and AT kits allowed for successful mtDNA control region sequencing from 1µl of a 1:100 dilution of the WGA product.
12. Whole genome amplification of 1-6pg genomic DNA using a mtDNA specific WGA kit – the REPLI-g Mitochondrial DNA kit – allowed for successful mtDNA control region sequencing from 1µl of a 1:100 dilution (1pg starting template) or 1:1000 dilution (6pg starting template) of the WGA product.
13. Mitochondrial control region sequencing of LMD cells showed limited success. Prior amplification of LMD cells with the mtDNA specific WGA kit did not improve mtDNA control region sequencing. Such results are likely due to inefficient extraction techniques or inherent inhibitors in the samples.

7.8 Summary

In conclusion this work has shown that improvements can be made to current LTDNA analysis techniques to provide additional information to stakeholders for forensic investigation or legal purposes. Simply concentrating a LTDNA sample for amplification can provide a considerable amount of additional information compared to replicate analysis methods. Implementation of WGA or linear Pre-PCR amplification can increase the amount of template available for traditional STR analysis. However, some stochastic variation is still observed with these techniques therefore further improvement is still necessary.

Difficulties with mixture analysis can be avoided if LMD is implemented to isolate single cells for analysis. WGA of single cells would allow for immortalisation of the genome so that numerous reactions could be performed. However optimised extraction methodologies must be developed to ensure that LMD cells can be successfully amplified with WGA chemistries.

Mitochondrial DNA control region sequencing can be successfully performed using substantially less starting template than traditional autosomal STR analysis. Frequency estimates of mtDNA haplotypes may not be as discriminating as rarity statistics provided for complete STR profiles. However, depending on the size of the mtDNA database(s) used for comparison, a complete mtDNA sequence could potentially be more discriminating than a partial STR profile obtained from limited stating template. Whole mtDNA genome sequencing using massively parallel sequencing would provide considerably more information than control region sequencing. Mitochondrial WGA could allow for LTDNA to become a viable sample type for such emerging technologies.

CHAPTER 8

REFERENCES

1. Wambaugh J. The Blooding. New York (NY): Bantam Books;1989.
2. Gill P, Whitaker J, Flaxman C, Brown N, Buckleton J. An investigation of the rigor of interpretation rules for STRs derived from less than 100pg of DNA. Forensic Sci Int. 2000; 112:17-40.
3. Caragine T, Mikulasovich R, Tamariz S, Bajda E, Sebertyen J, Baum H, et al. Validation of testing and interpretation protocols for low template DNA samples using AmpFlSTR Identifiler. Croat Med J. 2009; 50:250-67.
4. Benschop CCG, van der Beek CP, Meiland HC, van Gorp AGM, Westen AA, Sijen T. Low template STR typing: Effect if replicate number and consensus method on genotyping reliability and DNA database search results. Forensic Sci Int. Genet 2011; 5(4):316-28.
5. Forster L, Thompson J, Kutranov S. Direct comparison of post-28-cycle PCR purification and modified capillary electrophoresis methods with 34-cycle "low copy number" (LCN) method for analysis of trace forensic DNA samples. Forensic Sci Int. Genet 2008; 2:318-28.
6. Petricevic S, Whitaker J, Buckleton J, Viniter S, Patel J, Simon P, et al. Validation and development of interpretation guidelines for low copy number (LCN) DNA profiling in New Zealand using AmpFlSTR SGM Plus multiplex. Forensic Sci Int. Genet 2010; 4(5):305-10.
7. Moretti TR, Baumstark AL, Defenbaugh DA, Keys KM, Smerick JB, Budowle B. Validation of short tandem repeats (STRs) for forensic usage: performance testing of fluorescent multiplex STR systems and analysis of authentic and simulated forensic samples. J Forensic Sci. 2001; 46(3):647–60.
8. Collins PJ, Hennessy LK, Leibelt CS, Roby RK, Reeder DJ, Foxall PA. Developmental validation of a single-tube amplification of the 13 CODIS STR loci, D2S1338, D19S433 and amelogenin: the AmpFlSTR Identifiler PCR Amplification Kit. J Forensic Sci. 2004; 49(6):1265-77.
9. Cotton EA, Allsop RF, Guest JL, Frazier RR, Koumi P, Callow IP, et al. Validation of the AmpFlSTR SGM plus system for use in forensic casework. Forensic Sci Int. 2000; 112:151-61.
10. Krenke BE, Terba A, Anderson SJ, Buel E, Culhane S, Finis CJ, et al. Validation of a 16 locus fluorescent multiplex system. J Forensic Sci. 2002; 47(4):733-85.

11. Holt CL, Buoncristiani M, Wallin JM, Nguyen T, Lazaruk KD, Walsh PS. TWGDAM validation of AmpFlSTR™ PCR amplification kits for forensic DNA casework. J Forensic Sci. 2002; 47(1):66–96.
12. Tucker VC, Hopwood AJ, Sprecher CJ, McLaren RS, Rabbach DR, Ensenberger MG, et al. Developmental validation of the PowerPlex® ESI 16 and PowerPlex® ESI 17 systems: STR multiplexes for the new European standard. Forensic Sci Int Genet. 2011; 5:436-48.
13. Green RL, Lagace RE, Oldroyd NJ, Hennessy LK, Mulero JJ. Developmental validation of the AmpFlSTR NGM SElect™ PCR Amplification Kit: A next-generation STR multiplex with the SE33 locus. Forensic Sci Int Genet. 2013; 41-51.
14. Findlay I, Taylor A, Quirke P, Frazier R, Urquhart A. DNA fingerprinting from single cells. Nature. 1997; 389:555-6.
15. Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, et al. The sequence of the human genome. Science. 2001; 291:1304:51.
16. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, et al. Initial sequencing and analysis of the human genome. Nature. 2001; 409(6822):860-921.
17. Budowle B, van Daal A. Extracting evidence from forensic DNA analysis: Future molecular biology directions. BioTechniques 2009; 46(5):339-50.
18. Mullis K, Faloona F, Scharf S, Saiki R, Erlich H. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harb Symp Quant Biol. 1986; 51(1):263-73.
19. Butler JM. Short tandem repeat typing technologies used in human identity testing. BioTechniques. 2007; 43(Suppl):Sii-Sv.
20. Butler JM, Buel E, Crivellente F, McCord BR. Forensic DNA typing by capillary electrophoresis using the ABI Prism 310 and 3100 genetic analysers for STR analysis. Electrophoresis. 2004; 25:1397–412.
21. Scientific Working Group on DNA Analysis Methods (SWGDAM). SWGDAM interpretation guidelines for autosomal STR typing by forensic DNA testing laboratories [document on the Internet]. 2010 [cited 2013 Sept 30]. Available from: http://www.fbi.gov/about-us/lab/biometric-analysis/codis/swgdam.pdf
22. Budowle B, Onorato AJ, Callaghan TF, Della Manna A, Gross AM, Guerrieri RA, et al. Mixture interpretation: Defining the relevant features for guidelines

for the assessment of mixed DNA profiles in forensic casework. J Forensic Sci. 2009; 54(4):810-21.

23. Budowle B, Eisenberg AJ, & van Daal A. Validity of low copy number typing and applications to forensic science. Croat Med J. 2009; 50:207-17.
24. Gill P, Puch-Solis R, Curran J. The low template DNA (stochastic) threshold – Its determination relative to risk analysis for national DNA databases. Forensic Sci Int Genet. 2009; 3:104-11.
25. Schlotterer C, Tautz D. Slippage synthesis of simple sequence DNA. Nucleic Acids Res. 1992; 20(2):211-15.
26. Walsh PS, Fildes NJ, Reynolds R. Sequence analysis and characterisation of stutter products at the tetranucleotide repeat locus vWA. Nucleic Acids Res. 1996; 24(14):2807-12.
27. Gill P, Brenner CH, Buckleton JS, Carracedo A, Krawczak M, Mayr WR, et al. DNA commission of the International Society of Forensic Genetics: Recommendations on the interpretation of mixtures. Forensic Sci Int. 2006; 160:90-101.
28. Van Oorschot R, Jones M. DNA fingerprints from fingerprints. Nature 1997; 387:767.
29. Gill P, Buckleton J. Low copy number typing – Where next? Forensic Sci Int: Genet Suppl Ser. 2009; 2(1):553-5.
30. Petricevic SF, Bright J, Cockerton SL. DNA profiling of trace DNA recovered from bedding. Forensic Sci Int. 2006; 159(1):21-6.
31. Budowle B, Hobson DL, Smerick JB, Smith JAL. Low copy number – Consideration and caution. In: Twelfth International Symposium on Human Identification; 2001 Oct 9-12; Biloxi, Mississippi. Promega Corporation; 2001.
32. Roeder AD, Elsmore P, Greenhalgh M, McDonald A. Maximising DNA profiling success from sub-optimal quantities of DNA: A stages approach. Forensic Sci Int Genet. 2007; 3:128-37.
33. Caddy R, Taylor G, Linacre A. A review of the science of Low Template DNA analysis. [document on the internet]. Home Office, United Kingdom; 2008 [cited 2013 Sept 30]. Available from: http://www.homeoffice.gov.uk/publications/police/operational-policing/Review_of_Low_Template_DNA_1.pdf?view=Binary.

34. Castella V, Mangin P. DNA profiling success from and relevance of 1739 contact stains from casework. Forensic Sci Int: Genet Suppl Ser. 2008; 1(1):405-7.
35. Alessandrini F, Cecati M, Pesaresi M, Turchi C, Carle F, Tagliabracci A. Fingerprints as evidence for a genetic profile: Morphological study on fingerprints and analysis of exogenous and individual factors affecting DNA typing. J Forensic Sci. 2003; 48(3):586-92.
36. Aditya S, Sharma AK, Bhattacharyya CN, Chaudhuri K. Generating STR profile from "Touch DNA". J Forensic Leg Med. 2011; 18(7):295-8.
37. Prinz M, Schiffner L, Sebestyen JA, Bajda E, Tamariz J, Shaler RC, et al. Maximization of STR DNA typing success for touched objects. Int Congress Series. 2006; 1228:651-3.
38. Kita T, Yamaguchi H, Yokoyama M, Tanaka T, Tanaka N. Morphological study of fragmented DNA on touched objects. Forensic Sci Int Genet. 2008; 3:32-6.
39. Sewel J, Quinones I, Ames C, Multaney B, Curtis S, Seeboruth H, et al. Recovery of DNA and fingerprints from touched documents. Forensic Sci Int Genet. 2008; 2(4):281-5.
40. Balogh MK, Burger J, Bender K, Schneider PM, Alt KW. STR genotyping and mtDNA sequencing of latent fingerprints on paper. Forensic Sci Int. 2003; 137:188-95.
41. Bright J, Petricevic SF. Recovery of trace DNA and its application to DNA profiling of shoe insoles. For Sci Int. 2004; 145:7-12.
42. Irwin JA, Edson SM, Loreille O, Just RS, Barritt SM, Lee DA, et al. DNA identification of "Earthquake McGoon" 50 years post mortem. J Forensic Sci. 2007; 52(5):1115-8.
43. Amory S, Keyser C, Crubezy E, Ludes B. STR typing of ancient DNA extracted from hair shafts of Siberian mummies. Forensic Sci Int. 2007; 116:218-29.
44. Smith P, Ballantyne J. Simplified low-copy-number DNA analysis by post-PCR purification. J Forensic Sci. 2007; 52(4):820-9.
45. Weiler NEC, Matai AS, Sijen T. Extended PCR conditions to reduce drop-out frequencies in low template STR typing including unequal mixtures. Forensic Sci Int Genet. 2012; 6(1):102-7.

46. Hua Zhang A, Bum Seo S, Yi JA, Yeon Kim H, Deok Lee S. Modification of a commercially available kit for the improvement of PCR efficiency. Hum Biol. 2010; 82(3):343-51.
47. Westen AA, Nagel JHA, Benschop CCG, Weiler NEC, de Jong BJ, Sijen T. Higher capillary electrophoresis injection settings as an efficient approach to increase the sensitivity of STR typing. J Forensic Sci. 2009; 54(3):591-8.
48. Buckleton J. Validation issues around DNA typing of low level DNA. Forensic Sci Int Genet. 2009; 3:253-60.
49. Alonso A, Martin P, Albarran C, Garcia P, Primorac D, Garcia O, et al. Specific quantification of human genomes from low copy number DNA samples in forensic and ancient DNA studies. Croat Med J. 2003; 44(3):273-80.
50. Linacre, A. Review of Low Template DNA typing. Forensic Sci Int: Genet Suppl Ser. 2009; 2(1):549-50.
51. Crown Prosecution Service. Low Copy Number DNA testing in the criminal justice system. [document on the internet]. The Crown Prosecution Service; 2009 [cited 2013 Sept 30]. Available from: http://www.cps.gov.uk/publications/prosecution/lcn_testing.html.
52. Benschop C, Haned H, Sijen T. Consensus and pool profiles to assist in the analysis and interpretation of complex low template DNA mixtures. Int J Legal Med. 2013; 127(1):11-23.
53. Bright JA, Gill P, Buckleton J. Composite profiles in DNA analysis. Forensic Sci Int Genet. 2012; 6(3):317-21.
54. Gill P, Buckleton J. A universal strategy to interpret DNA profiles that does not require a definition of low-copy-number. Forensic Sci Int Genet. 2010; 4(4):221-7.
55. Grisedale K, van Daal A. Comparison of STR profiling from low template DNA extracts with and without the consensus profiling method. Investig Genet. 2012; 3:14.
56. Balding DJ, Buckleton J. Interpreting low template DNA profiles. Forensic Sci Int Genet. 2009; 4:1-10.
57. Gill P, Kirkham A, Curran J. LoComatioN: a software tool for the analysis of low copy number DNA profiles. Forensic Sci Int. 2006; 166:128-138.

58. Curran J, Gill P, Bill MR. Interpretation of repeat measurement DNA evidence allowing for multiple contributors and population substructure. Forensic Sci Int. 2005; 148:47-53.
59. Mitchell AA, Tamariz J, O'Connell K, Ducasse N, Budimlija Z, Prinz M, et al. Validation of a DNA mixture statistics tool incorporating allelic drop-out and drop-in. Forensic Sci Int Genet. 2012; 6:749-761.
60. Haned H. Forensim: An open-source initiative for the evaluation of statistical methods in forensic genetics. Forensic Sci Int Genet. 2011; 5:265-268.
61. Perlin MW, Legler MM, Spencer CE, Smith JL, Allan WP, Belrose JL, et al. Validating TrueAllele® DNA Mixture Interpretation. J Forensic Sci. 2011; 56:1430-1447.
62. Nurit B, Anat G, Michal S, Lilach F, Maya F. Evaluating the prevalence of DNA mixtures found in fingernail samples from victims and suspects in homicide cases. Forensic Sci Int Genet. 2011; 5(5):532-7.
63. Benschop CC, Haned H, de Blaeij TJ, Mullenbroek AJ, Sijen T. Assessment of mock cases involving complex low template DNA mixtures: A descriptive study. Forensic Sci Int Genet. 2012; 6(6):697-707.
64. Kelly H, Bright JA, Curran J, Buckleton J. The interpretation of low level DNA mixtures. Forensic Sci Int Genet. 2012; 6(2):191-7.
65. Pfeifer CM, Klein-Unseld R, Klintschar M, Wiegand P. Comparison of different interpretation strategies for low template DNA mixtures. Forensic Sci Int Genet. 2012; 6(6):716-22.
66. Juusola J, Ballantyne J. Messenger RNA profiling: a prototype method to supplant conventional methods for body fluid identification. Forensic Sci Int. 2003; 135(2):85-96.
67. Lindenbergh A, de Pagter M, Ramdayal G, Visser M, Zubakov D, Kayser M, et al. A multiplex (m)RNA-profiling system for the forensic identification of body fluids and contact traces. Forensic Sci Int Genet. 2012; 6(5):565-77.
68. Hanson EK, Lubenow H, Ballantyne J. Identification of forensically relevant body fluids using a panel of differentially expressed microRNAs. Anal Biochem. 2009; 387(2):303-14.
69. Frumkin D, Wasserstrom A, Budowle B, Davidson A. DNA methylation-based forensic tissue identification. Forensic Sci Int Genet. 2011; 5(5):517-24.

70. The Queen v Sean Hoey. Neutral Citation No. [2007] NICC 49, 2007. [cited 2013 Sept 30] Available from: http://www.xproexperts.co.uk/newsletters/feb08/R%20v%20Hoey.pdf.
71. McCartney C. LCN DNA: Proof beyond reasonable doubt? Nat Rev Genet. 2008; 9(5):325.
72. Gilder J, Koppl R, Kornfield I, Krane D, Mueller L, Thompson W. Comments on the review of low copy number testing. Int J Legal Med. 2009; 123(6):535-6.
73. Jamieson A, Bader S. Press release on behalf of the Forensic Institute, Glasgow regarding the Caddy Review of Low Template DNA. [document on the internet]. Forensic Institute, Glasgow; 2008 [cited 2013 Sept 30]. Available from: http://www.denverda.org/DNA_Documents/Forensic%20Institute,%20Glasgow%20Press%20release.pdf.
74. Hill CR, Kline MC, Coble MD, Butler JM. Characterization of 26 MiniSTR Loci for Improved analysis of degraded DNA samples. J Forensic Sci. 2008; 53(1):73-80.
75. Lagoa AM, Magalhaes T, Pinheiro MF. Autosomic STR, Y-STR and miniSTR markers evaluation for genetic analysis of fingerprints. Forensic Sci Int: Genet Suppl Ser. 2008; 1(1):435-6.
76. Mulero JJ, Chang CW, Lagac RE, Wang DY, Bas JL, McMahon TP, et al. Development and validation of the AmpFlSTR MiniFilerTM PCR Amplification Kit: A MiniSTR multiplex for the analysis of degraded and/or PCR inhibited DNA. J Forensic Sci. 2008; 53(4):838-52.
77. Raymond JJ, van Oorschot RAH, Walsh SJ, Roux C. Trace DNA analysis: Do you know what your neighbour is doing? A multi-jurisdictional survey. Forensic Sci Int Genet. 2008; 2(1):19-28.
78. Espina V, Wulfkuhle JD, Calvert VS, Van Meter A, Zhou W, Loukos G, et al. Laser-Capture Microdissection. Nat Protoc. 2006; 1(2):586-603.
79. Sanders CT, Sanchez N, Ballantyne J, Peterson DA. Laser microdissection separation of pure spermatozoa from epithelial cells for short tandem repeat analysis. J Forensic Sci. 2006; 51(4):748-57.
80. Budimlija ZM, Lechpammer M, Popiolek D, Fogt F, Prinz M, Bieber FR. Forensic application of laser capture microdissection: Use in DNA-based parentage testing and platform validation. Croat Med J. 2005; 46(4):549-55.

81. Anoruo B, van Oorschot RAH, Mitchell J, Howells D. Isolating cells from non-sperm cellular mixtures using the PALM microlaser micro dissection system. Forensic Sci Int. 2007; 173(2-3):93-6.
82. Di Martino D, Giuffre G, Staiti N, Simone A, Todaro P, Saravo L. Laser microdissection and DNA typing of cells from single hair follicles. Forensic Sci Int. 2004; 146S:S155-7.
83. Robino C, Barilaro MR, Gino S, Chiarle R, Palestro G, Torre C. Incestuous paternity detected by STR-typing of chorionic villi isolated from archival formalin-fixed paraffin-embedded abortion material using laser microdissection. J Forensic Sci. 2006; 51(1)90-2.
84. Di Martino D, Giuffre G, Staiti N, Simone A, LeDonne M, Saravo L. Single sperm cell isolation by laser microdissection. Forensic Sci Int. 2004; 146S:S151-3.
85. Di Martino D, Giuffre G, Staiti N, Simone A, Sippelli G, Tuccari G, et al. LMD as a forensic tool in a sexual assault casework: LCN DNA typing to identify the responsible. Int Congress Series. 2006; 1288:571-3.
86. Anslinger K, Mack B, Bayer B, Rolf B, Eisenmenger W. Digoxigenin labelling and laser capture microdissection of male cells. Int J Legal Med. 2005; 119(6):374-7.
87. Murray C, McAlister C, Elliot K. Use of fluorescence in situ hybridisation and laser microdissection to isolate male non-sperm cells in cases of sexual assault. Int Congress Series. 2006; 1288:622-4.
88. Meredith M, Bright JA, Cockerton S, Vintiner S. Development of a one-tube extraction and amplification method for DNA analysis of sperm and epithelial cells recovered from forensic samples by laser microdissection. Forensic Sci Int Genet. 2012; 6(1):91-6.
89. Thorpe SM, Pedersen NA, Seddon TJ, Prince DV, Goucher MJ, van Oorschot RAH. Laser microdissection: Checking that the dissected cells are recovered for DNA extraction. Forensic Sci Int: Genet Suppl Ser. 2011; 3:e145-6.
90. Thorpe SM, Prince DV, van Oorschot RAH. Comparison of extraction methods for spermatozoa recovered using laser microdissection. Forensic Sci Int: Genet Suppl Ser. 2011; 3:e147-8.
91. Vandewoestyne M, Deforce D. Laser capture microdissection for forensic DNA analysis. Forensic Sci Int: Genet Suppl Ser. 2011; 3:e117-8.

92. Staiti N, Giuffe G, Di Martino D, Simone A, Sippelli G, Tuccari G, et al. Molecular analysis of genomic low copy number DNA extracted from laser-microdissected cells. Int Congress Series. 2006; 1288:568-70.
93. Yamamoto Y, Hara M, Takada A, Kido A, Miyazaki T, Saito K. STR loci analysis of buccal cavity cells captured by laser microdissection. Int Congress Series. 2006; 1288:666-8.
94. Frumkin D, Wasserstrom A, Itzkovitz S, Harmelin A, Rechavi G, Shapiro E. Amplification of multiple genomic loci from single cells isolated by laser micro-dissection of tissues. BMC Biotechnol. 2008; 8:17.
95. Aaltonen KE, Ebbesson A, Wigerup C, Hedenfalk I. Laser capture microdissection (LCM) and whole genome amplification (WGA) of DNA from normal breast tissue - optimization for genome wide array analyses. BMC Res Notes. 2011; 4:69.
96. Murphy SJ, Cheville JC, Zarei S, Johnson SH, Sikkink RA, Kosari F, et al. Mate pair sequencing of whole-genome-amplified DNA following laser capture microdissection of prostate cancer. DNA Res. 2012; 19(5):395-406.
97. Zhang L, Cui X, Schmitt K, Hubert R, Navidit W, Arnheim N. Whole genome amplification from a single cell: Implications for genetic analysis. Proc Natl Acad Sci USA. 1992; 89:5847-51.
98. Telenius H, Carter NP, Bebb CE, Nordenskjold M, Ponder BA, Tunnacliffe A. Degenerate oligonucleotide-primed PCR: general amplification of target DNA by a single degenerate primer. Genomics. 1992; 13(3):718-25.
99. Grothues D, Cantor CR, Smith CL. PCR amplification of megabase DNA with tagged random primers (T-PCR). Nucleic Acids Res. 1993; 21(5):1321-2.
100. Lengauer C, Green ED, Cremer T. Fluorescence in Situ Hybridization of YAC Clones after Alu-PCR Amplification. Genomics. 1992; 13:826-8.
101. Wells D, Sherlock JK, Handyside AH, Delhanty JDA. Detailed chromosomal and molecular genetic analysis of single cells by whole genome amplification and comparative genomic hybridisation. Nucleic Acids Res. 1999; 27(4):1214-8.
102. Snabes MC, Chong SS, Subramanian SB, Kristjansson K, Disepio D, Hughes MR. Preimplantation single-cell analysis of multiple genetic loci by whole-genome amplification. Proc Natl Acad Sci USA. 1994; 91:6181-5.

103. Wells D, Delhanty JDA. Comprehensive chromosomal analysis of human preimplantation embryos using whole genome amplification and single cell comparative genomic hybridization. Mol Hum Reprod. 2000; 6(11):1055-62.
104. Cheung VG, Nelson SF. Whole genome amplification using a degenerate oligonucleotide primer allows hundreds of genotypes to be performed on less than one nanogram of genomic DNA. Proc Natl Acad Sci USA. 1996; 93:14676-9.
105. Hanson EK, Ballantyne J. Whole genome amplification strategy for forensic genetic analysis using single or few cell equivalents of genomic DNA. Anal Biochem. 2005; 346(2):246-57.
106. Dietmaier W, Hartmann A, Wallinger S, Heinmoller E, Kerner T, Endl E, et al. Multiple mutation analyses in single tumor cells with improved whole genome amplification. Am J Pathol. 1999; 154(1):83-95.
107. Kittler R, Stoneking M, Kayser M. A whole genome amplification method to generate long fragments from low quantities of genomic DNA. Anal Biochem. 2002; 300(2):237-44.
108. Bergen AW, Haque KA, Qi Y, Beerman MB, Garcia-Closas M, Rothman N, et al. Comparison of yield and genotyping performance of multiple displacement amplification and OmniPlex whole genome amplified DNA generated from multiple DNA sources. Hum Mutat. 2005; 26(3):262-70.
109. Sun G, Kaushal R, Pal P, Wolujewicz M, Smelser D, Cheng H, et al. Whole-genome amplification: relative efficiencies of the current methods. Leg Med (Tokyo). 2005; 7(5):279-86.
110. Barber A, Foran DR. The utility of whole genome amplification for typing compromised forensic samples. J Forensic Sci. 2006; 51(6):1344-9
111. Pinard R, de Winter A, Sarkis GJ, Gerstein MB, Tartaro KR, Plant RN et al. Assessment of whole genome amplification-induced bias through high-throughput, massively parallel whole genome sequencing. BMC Genomics. 2006; 7:216.
112. Lee JC, Tsai LC, Lai PY, Lee CC, Lin CY, Huang TY. Evaluating the performance of whole genome amplification for use in low template DNA typing. Med Sci Law. 2012; 52:223-8.

113. Maciejewska A, Jakubowska J, Pawłowski R. Whole genome amplification of degraded and nondegraded DNA for forensic purposes. Int J Legal Med. 2013; 127:309-19.
114. Spits C, Le Caignee C, DeRycke M, Van Haute L, Van Steirteghem A, Liebaers I, et al. Whole-genome multiple displacement amplification from single cells. Nat Protoc. 2006; 1(4):1965-70.
115. Ballantyne KN, van Oorschot RAH, Mitchell RJ. Comparison of two whole genome amplification methods for STR genotyping of LCN and degraded DNA samples. Forensic Sci Int. 2007; 166:35-41.
116. Lizardi PM, Huang X, Zhu Z, Bray-Ward P, Thomas DC, Ward DC. Mutation detection and single-molecule counting using isothermal rolling-circle amplification. Nat Genet. 1998; 19(3):225-32.
117. Dean FB, Hosono S, Fang L, Wu X, Farugi AF, Bray-Ward P, et al. Comprehensive human genome amplification using multiple displacement amplification. Proc Natl Acad Sci USA. 2002; 99(8):5261-6.
118. Blanco L, Bernad A, Lazharo JM, Martin G, Garmendia C, Salas M. Highly efficient DNA synthesis by the phage phi 29 DNA polymerase. Symmetrical mode of DNA replication. J Biol Chem. 1989; 25:8935-40.
119. Handyside AH, Robinson MD, Simpson R J, Omar MB, Shaw M, Grudzinskas JG, et al. Isothermal whole genome amplification from single and small numbers of cells: A new era for preimplantation genetic diagnosis of inherited disease. Mol Hum Reprod. 2004; 10(10):767-72.
120. Hellani A, Costeun S, Benkhalifa M, Tbakhi A, Sakati N, Al-Odaib A, et al. Multiple displacement amplification on single cell and possible PGD applications. Mol Hum Reprod. 2004; 10(11):847-52.
121. Jiang Z, Zhang X, Deka R, Jin L. Genome amplification of single sperm using multiple displacement amplification. Nucleic Acids Res. 2005; 33(10):e91.
122. Spits C, Le Caignec C, De Rycke M, Van Haute L, Van Steirteghem A, Liebaers I, et al. Optimization and evaluation of single-cell whole-genome multiple displacement amplification. Hum Mutat. 2006; 27(5):496-503.
123. Kumar G, Garnova E, Reagin M, Vidali A. Improved multiple displacement amplification with Φ29 DNA polymerase for genotyping of single human cells. BioTechniques. 2008; 44(7):879-90.

124. Schneider PM, Balogh K, Naveran N, Bogus M, Bender K, Lareu M, et al. Whole genome amplification—the solution for a common problem in forensic casework? Int Congress Series. 2004; 1261:24-6.
125. Balogh K, Borsting C, Sanchez Diz P, Thacker C, Syndercombe-Court D, Carracedo A, et al. Application of whole genome amplification for forensic analysis. Int Congress Series. 2006; 1288:725-27.
126. Lagoa AM, Magalhaes T, Pinheiro MF. Genetic analysis of fingerprints – Could WGA or nested-PCR be alternatives to the increase of PCR cycle number? Forensic Sci Int: Genetics Supplement Series. 2008; 1(1):48-9.
127. Ballantyne KN, van Oorschot RAH, Mitchell RJ, Koukoulas I. Molecular crowding increases the amplification success of multiple displacement amplification and short tandem repeat genotyping. Anal Biochem. 2006; 355:298-303.
128. Bexon K, Williams G. Evaluating the effect of multiple displacement amplification upon low-level multiple source DNA profiles. Int J Crim Investig. 2012; 2(2):103-10.
129. Ballantyne KN, van Oorschot RAH, Muharam I, van Daal A, Mitchell J. Decreasing amplification bias associated with multiple displacement amplification and short tandem repeat genotyping. Anal Biochem. 2007; 368(2):222-9.
130. Alsmadi O, Alkayal F, Monies D, Meyer BF. Specific and complete human genome amplification with improved yield achieved by phi29 DNA polymerase and a novel primer at elevated temperature. BMC Res Notes. 2009; 2:48.
131. Butler JM, Levin BC. Forensic applications of mitochondrial DNA. Trends Biotechnol. 1998; 16(4):158-62.
132. Holland MM, Fisher DL, Mitchell LG, Rodriquez WC, Canik JJ, Merril CR, et al. Mitochondrial DNA sequence analysis of human skeletal remains identification of remains from the Vietnam war. J Forensic Sci. 1993; 38(3):542-53.
133. Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, et al. Sequence and organization of the human mitochondrial genome. Nature. 1981; 290(5806):457-65.

134. Andrews RM, Kubacka I, Chinnery PF, Lightowlers RN, Turnbull DM, Howell N. Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. Nat Genet. 1999; 23(2):147.
135. Bogenhagen D, Clayton DA. The number of mitochondrial deoxyribonucleic acid genomes in mouse L and human HeLa cells. Quantitative isolation of mitochondrial deoxyribonucleic acid. J Biol Chem. 1974; 249(24):7991-5.
136. Budowle B, Bieber FR, Eisenberg AJ. Forensic aspects of mass disasters: Strategic considerations for DNA-based human identification. Leg Med (Tokyo). 2005; 7(4):230-43.
137. Sullivan KM, Hopgood R, Gill P. Identification of human remains by amplification and automated sequencing of mitochondrial DNA. Int J Legal Med. 1992; 105(2):83-6.
138. Gill P, Ivanov PL, Kimpton C, Piercy R, Benson N, Tully G, et al. Identification of the remains of the Romanov family by DNA analysis. Nat Genet. 1994; 6(2):130-5.
139. Holland, M. Fisher DL, Roby RK, Ruderman J, Bryson C, Weedn W. Mitochondrial DNA sequence analysis of human remains. Crime Lab Digest. 1995; 22(4):109-15.
140. Rousselet F, Mangin P. Mitochondrial DNA polymorphisms: A study of 50 French Caucasian individuals and application to forensic casework. Int J Legal Med. 1998; 111:292-8.
141. Nelson K, Melton T. Forensic Mitochondrial DNA analysis of 116 casework skeletal samples. J Forensic Sci. 2007; 52(3):557-61.
142. Wilson MR, DiZinno JA, Polanskey D, Replogle J, Budowle B. Validation of mitochondrial DNA sequencing for forensic casework analysis. Int J Legal Med. 1995; 108(2):68-74.
143. Allen M, Engstrom AS, Meyers, S, Handt O, Saldeen T, von Haeseler A, et al. Mitochondrial DNA sequencing of shed hairs and saliva on robbery caps: Sensitivity and matching probabilities. J Forensic Sci. 1998; 43(3):453-64.
144. Parson W, Parsons TJ, Scheithauer R, Holland MM. Population data for 101 Austrian Caucasian mitochondrial DNA d-loop sequences: Application of mtDNA sequence analysis to a forensic case. Int J Legal Med. 1998; 111:124-32.

145. Melton T, Nelson K. Forensic mitochondrial DNA analysis: Two years of commercial casework in the United States. Croat Med J. 2001; 42(3):298-303.
146. Erdem S, Altuncul H, Filoglu G, Olcen AM, Bulbul O. Sequencing of mtDNA HV1 and HV2 regions from samples with trace amount of DNA. Forensic Sci Int: Genet Suppl Ser. 2011; 3(1):e455-6.
147. Ginther C, Issel-Tarver L, King MC. Identifying individuals by sequencing mitochondrial DNA from teeth. Nat Genet. 1992; 2(2):135-8.
148. Bataille M, Crainic K, Leterreux M, Durigon M, de Mazancourt P. Multiplex amplification of mitochondrial DNA for human and species identification in forensic evaluation. Forensic Sci Int. 1999; 99(3):165-70.
149. Hopwood AJ, Mannucci A, Sullivan KM. DNA typing from human faeces. Int J Legal Med. 1996; 108(5):237-43.
150. Wilson MR, Stoneking M, Holland MM, DiZinno JA, Budowle B. Guidelines for the Use of Mitochondrial DNA Sequencing in Forensic Science. Crime Lab Digest. 1993; 20(4):68-77.
151. Bär W, Brinkmann B, Budowle B, Carracedo A, Gill P, Holland M, et al. DNA Commission of the International Society for Forensic Genetics: Guidelines for mitochondrial DNA typing. Int J Legal Med. 2000; 113:193-196.
152. Giles RE, Blanc H, Cann HM, Wallace DC. Maternal inheritance of human mitochondrial DNA. Proc Natl Acad Sci USA. 1980; 77(11):6715-9.
153. Cummins JM, Wakayama T, Yanagimachi R. Fate of microinjected sperm components in the mouse oocyte and embryo. Zygote. 1997; 5(4):301-8.
154. Budowle B, Allard MW, Wilson MR, Chakraborty R. Forensics and mitochondrial DNA: Applications, debates, and foundations. Annu Rev Genomics Hum Genet. 2003; 4:119–41.
155. Holland MM, Parsons TJ. Mitochondrial DNA sequence analysis – Validation and use for forensic casework. Forensic Sci Rev. 1999; 11(1):21-50.
156. Parsons TJ, Coble MD. Increasing the forensic discrimination of mitochondrial DNA testing through analysis of the entire mitochondrial DNA genome. Croat Med J. 2001; 42(3):304-9.
157. Budowle B, Eisenberg AJ, Gonzalez S, Planz JV, Sannes-Lowery KA, Hall TA, et al. Validation of mass spectrometry analysis of mitochondrial DNA. Forensic Sci Int: Genetics Supplement Series. 2009 2:527-8.

158. Brandstätter A, Parsons TJ, Parson W. Rapid screening of mtDNA coding region SNPs for the identification of west European Caucasian haplogroups. Int J Legal Med. 2003; 117:291-8.
159. Quintans B, Alvarez-Iglesias V, Salas A, Phillips C, Lareu MV, Carracedo A. Typing of mitochondrial DNA coding region SNPs of forensic and anthropological interest using SNaPshot minisequencing. Forensic Sci Int. 2004; 140:251-7.
160. Vallone PM, Just RS, Coble MD, Butler JM, Parsons TJ. A multiplex allele-specific primer extension assay for forensically informative SNPs distributed throughout the mitochondrial genome. Int J Legal Med. 2004; 118(3):147-57.
161. Coble MD, Just RS, O'Callaghan JE, Letmanyi IH, Peterson CT, Irwin JA, et al. Single nucleotide polymorphisms over the entire mtDNA genome that increase the power of forensic testing in Caucasians. Int J Legal Med. 2004; 118:137-46.
162. Salas A, Carracedo A, Macaulay V, Richards M, Bandelt HJ. A practical guide to mitochondrial DNA error prevention in clinical, forensic, and population genetics. Biochem Biophys Res Commun. 2005; 335(3):891-9.
163. Köhnemann S, Sibbing U, Pfeiffer H, Hohoff C. A rapid mtDNA assay of 22 SNPs in one multiplex reaction increases the power of forensic testing in European Caucasians. Int J Legal Med. 2008; 122(6):517-23.
164. Fendt L, Zimmermann B, Daniaux M, Parson W. Sequencing strategy for the whole mitochondrial genome resulting in high quality sequences. BMC Genomics. 2009; 10:139.
165. Tang S, Huang T. Characterization of mitochondrial DNA heteroplasmy using a parallel sequencing system. BioTechniques. 2010; 48(4):287-95.
166. Gunnarsdottir ED, Li M, Bauchet M, Finstermeier K, Stoneking M. High-throughput sequencing of complete human mtDNA genomes from the Philippines. Genome Res. 2011; 21:1-11.
167. Parson W, Strobl C, Huber G, Zimmermann B, Gomes SM, Souto L, et al. Evaluation of next generation mtGenome sequencing using the Ion Torrent Personal Genome Machine (PGM). Forensic Sci Int Genet. 2013; 7(5):543-9.
168. Strom CM, Rechitsky S. Use of nested PCR to identify charred human remains and minute amounts of blood. J Forensic Sci. 1998; 43(3):696-700.
169. Nelson JR, Grossmann GA, Duthie RS, Shah SJ, Hell RC, Inventors; General Electric Company, assignee. Methods and kits for reducing non-specific nucleic

acid amplification. United States patent application US20130210078. August 15, 2013.

170. Nelson JR, Duthie RS, Grossmann GA, inventors; General Electric Company, assignee. Contamination-free reagents for nucleic acid amplification. United States patent application US20091055859. June 18, 2009.
171. Online Mendelian Inheritance in Man (OMIM). 203200 Albinism, Oculocutaneous, Type II; OCA2. [document on the internet]. 1986 [updated 2013 Sept 12; cited 2013 Sept 30]. Available from: http://omim.org/entry/203200.
172. Hughes-Stamm S. DNA typing methods for highly degraded samples. PhD [thesis]. Robina (QLD): Bond University; 2012.
173. Promega Corporation. PowerPlex® ESI 16 System. [document on the internet]. 2013 [updated 2013 May; cited 2013 Sept 30]. Available from: http://www.promega.com/~/media/Files/Resources/Protocols/Technical%20Manuals/101/PowerPlex%20ESI%2016%20System%20Protocol.pdf.
174. Life Technologies. AmpFlSTR® Identifiler® PCR Amplification Kit. [document on the internet]. 2012 [cited 2013 Sept 30]. Available from: http://tools.lifetechnologies.com/content/sfs/manuals/cms_041201.pdf.
175. GE Healthcare. Illustra GenomiPhi V2 DNA Amplification Kit. [document on the internet]. 2006 [cited 2013 Sept 30]. Available from: http://cichlid.umd.edu/cichlidlabs/protocols/Basic/GPHI_V2_25660030_revB.pdf.
176. Maragh S, Jakupciak JP, Wagner PD, Rom WN, Sidransky D, Srivastava S, et al. Multiple strand displacement amplification of mitochondrial DNA from clinical samples. BMC Med Genet. 2008; 9:7.
177. Hall TA, BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser. 1999; 41:95-8.
178. Life Technologies. Preparing Mitochondrial Genome Libraries Using Long Amplicons (8kb). [document on the internet]. 2012 [updated 2013 Aug 1; cited 2013 Sept 30]. Available from: http://ioncommunity.lifetechnologies.com/docs/DOC-6611.